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(54) Title: PRODUCTION OF HUMANIZED ANTIBODIES IN TRANSGENIC ANIMALS

(57) **Abstract:** This invention relates to humanized antibodies and antibody preparations produced from transgenic non-human animals. The non-human animals are genetically engineered to contain one or more humanized immunoglobulin loci which are capable of undergoing gene rearrangement and gene conversion in the transgenic non-human animals to produce diversified humanized immunoglobulins. The present invention further relates to novel sequences, recombination vectors and transgenic vectors useful for making these transgenic animals. The humanized antibodies of the present invention have minimal immunogenicity to humans and are appropriate for use in the therapeutic treatment of human subjects.

Production of Humanized Antibodies In Transgenic Animals

Field of the Invention

This invention relates to humanized antibodies produced from transgenic non-human animals. The non-human animals are genetically engineered to contain one or more humanized immunoglobulin loci which are capable of undergoing gene rearrangement and gene conversion in the transgenic non-human animals to produce diversified humanized immunoglobulins. The present invention further relates to novel sequences, recombination vectors and transgenic vectors useful for making these transgenic animals. The humanized antibodies of the present invention have minimal immunogenicity to humans and are appropriate for use in the therapeutic treatment of human subjects.

Background of the Invention

The therapy of infectious diseases caused by bacteria, fungi, virus and parasites is largely based on chemotherapy. However, the emergence of drug-resistant organisms requires the continuous development of new antibiotics. Therapies of patients with malignancies and cancer are also based on chemotherapy. However, many of these therapies are ineffective and the mortality of diseased patients is high. For both infectious diseases and cancer, improved and innovative therapies are needed.

Therapy of steroid resistant rejection of transplanted organs requires the use of biological reagents (monoclonal or polyclonal antibody preparations) that reverse the ongoing alloimmune response in the transplant recipient. The major problem of antibody preparations obtained from animals is the intrinsic immunogenicity of non-human immunoglobulins in human patients. In order to reduce the immunogenicity of non-human antibodies, genetic engineering of individual antibody genes in animals has been proposed. In particular, it has been shown that by fusing animal variable (V) region exons with human constant (C) region exons, a chimeric antibody gene can be obtained. However, this approach may only eliminate the immunogenicity caused by the non-human

Fc region, while the remaining non-human Fab sequences may still be immunogenic. In another approach, human immunoglobulin genes for both, heavy and light chain immunoglobulins have been introduced into the genome of mice. While this genetic engineering approach resulted in the expression of human immunoglobulin polypeptides in genetically engineered mice, the level of human immunoglobulin expression is low. This may be due to species-specific regulatory elements in the immunoglobulin loci that are necessary for efficient expression of immunoglobulins. As demonstrated in transfected cell lines, regulatory elements present in human immunoglobulin genes may not function properly in non-human animals.

Several regulatory elements in immunoglobulin genes have been described. Of particular importance are enhancers downstream (3') of heavy chain constant regions and intronic enhancers in light chain genes. In addition, other, yet to be identified, control elements may be present in immunoglobulin genes. Studies in mice have shown that the membrane and cytoplasmic tail of the membrane form of immunoglobulin molecules play an important role in expression levels of human-mouse chimeric antibodies in the serum of mice homozygous for the human Cγ1 gene. Therefore, for the expression of heterologous immunoglobulin genes in animals it is desirable to replace sequences that contain enhancer elements and exons encoding transmembrane (M1 exon) and cytoplasmic tail (M2 exon) with sequences that are normally found in the animal in similar positions.

The introduction of human immunoglobulin genes into the genome of mice resulted in expression of a diversified human antibody repertoire in genetically engineered mice. In both mice and humans, antibody diversity is generated by gene rearrangement. This process results in the generation of many different recombined V(D)J segments encoding a large number of antibody molecules with different antigen binding sites. However, in other animals, like rabbits, pigs, cows and birds, antibody diversity is generated by a substantially different mechanism called gene conversion. For example, it is well established that in rabbit and chicken, VDJ rearrangement is very limited (almost 90% of immunoglobulin is generated with the 3'proximal VH1 element) and antibody diversity is generated by gene conversion and hypermutation. In contrast, mouse and

human gene conversion occurs very rarely, if at all. Therefore, it is expected that in animals that diversify antibodies by gene conversion a genetic engineering approach based on gene rearrangement will result in animals with low antibody titers and limited antibody diversity. Thus, the genetic engineering of large animals for the production of non-immunogenic antibody preparations for human therapy requires alternative genetic engineering strategies.

Relevant Literature

The use of polyclonal antibody preparations for the treatment of transplant rejection was recently reviewed by N. Bonnefoy-Berard et al., *J Heart Lung Transplant* 1996; 15(5): 435-442; C. Colby et al., *Ann Pharmacother* 1996; 30(10):1164-1174; M.J. Dugan et al., *Ann Hematol* 1997; 75(1-2):41-46. The use of polyclonal antibody therapies for autoimmune diseases has been described by W. Cendrowski, *Boll Ist Sieroter Milan* 1997; 58(4):339-343; L.K. Kastrukoff et al., *Can J Neurol Sci* 1978; 5(2):175-178; J.E. Walker et al., *J Neurol Sci* 1976; 29(2-4):303-309. The depletion of fat cells using antibody preparations has been described by L. De Clercq et al., *J Anim Sci* 1997; 75(7):1791-1797; J.T. Wright et al., *Obes Res* 1995; 3(3):265-272.

Regulatory elements in immunoglobulin genes have been described by Bradley et al. (1999), *Transcriptional enhancers and the evolution of the IgH locus*; Lauster, R. et al., *Embo J* 12: 4615-23 (1993); Volgina et al., *J Immunol* 165:6400 (2000); Hole et al., *J Immunol* 146:4377 (1991).

Antibody diversification by gene conversion in chicken and rabbit has been described by Bucchini et al., *Nature* 326: 409-11 (1987); Knight et al., *Advances in Immunology* 56: 179-218 (1994); Langman et al., *Res Immunol* 144: 422-46 (1993). The generation of mice expressing human-mouse chimeric antibodies has been described by Pluschke et al., *Journal of Immunological Methods* 215: 27-37 (1998). The generation of mice expressing human-mouse chimeric antibodies with mouse derived membrane and cytoplamic tails has been described by Zou et al., *Science* 262: 1271-1274 (1993); Zou et al. *Curr Biol* 4: 1099-1103. The generation of mice expressing human immunoglobulin polypeptides has been described by Bruggemann et al. *Curr Opin Biotechnol* 8(4): 455-8 (1997); Lonberg et al. *Int Rev Immunol* 13(1):65-93 (1995); Neuberger et al., *Nature* 338:

350-2 (1989). Generation of transgenic mice using a BAC clone has been described by Yang et al., *Nat Biotechnol* 15: 859-65 (1997).

The generation of transgenic rabbits has been described by Fan, J. et al., Pathol Int 49: 583-94 (1999); Brem et al., *Mol Reprod Dev* 44: 56-62 (1996). Nuclear transfer cloning of rabbits has been described by Stice et al., *Biology of Reproduction* 39: 657-664 (1988). Rabbits with impaired immunoglobulin expression have been described by McCartney-Francis et al., *Mol Immunol* 24: 357-64 (1987); Allegrucci, et al., *Eur J Immunol* 21: 411-7 (1991).

The production of transgenic chicken has been described by Etches et al., *Methods in Molecular Biology* 62: 433-450; Pain et al., *Cells Tissues Organs* 1999; 165(3-4): 212-9; Sang, H., "Transgenic chickens--methods and potential applications", *Trends Biotechnol 12:415* (1994); and in WO 200075300, "Introducing a nucleic acid into an avian genome, useful for transfecting avian blastodermal cells for producing transgenic avian animals with the desired genes, by directly introducing the nucleic acid into the germinal disc of the egg".

Agammaglobulinemic chicken have been described by Frommel et al., *J Immunol* 105(1): 1-6 (1970); Benedict et al., *Adv Exp Med Biol* 1977; 88(2): 197-205.

The cloning of animals from cells has been described by T. Wakayama et al., *Nature* 1998; 394:369-374; J.B. Cibelli et al., *Science* 280:1256-1258 (1998); J.B. Cibelli et al., *Nature Biotechnology* 1998; 16:642-646; A. E. Schnieke et al., *Science* 278: 2130-2133 (1997); K.H. Campbell et al., *Nature* 380: 64-66 (1996).

Production of antibodies from transgenic animals is described in U.S. Patent No. 5,814,318, No. 5,545,807 and No. 5,570,429. Homologous recombination for chimeric mammalian hosts is exemplified in U.S. Patent No. 5,416,260. A method for introducing DNA into an embryo is described in U.S. Patent No. 5,567,607. Maintenance and expansion of embryonic stem cells is described in U.S. Patent No. 5,453,357.

The mechanisms involved in the diversification of the antibody repertoire in pigs, sheep and cows are reviewed in Butler, J. E. (1998), "Immunoglobulin diversity, B-cell and antibody repertoire development in large farm animals", *Rev Sci Tech 17:43.* Antibody diversification in sheep is described in Reynaud, C. A., C. Garcia, W. R. Hein,

and J. C. Weill (1995), "Hypermutation generating the sheep immunoglobulin repertoire is an antigen-independent process", *Cell 80:115*; and Dufour, V., S. Malinge, and F. Nau. (1996), "The sheep Ig variable region repertoire consists of a single VH family", *J Immunol 156:2163*.

Summary of the Invention

One embodiment of the present invention provides humanized antibodies (humanized immunoglobulins) having at least a portion of a human immunoglobulin polypeptide sequence.

The humanized antibodies of the present invention are made from transgenic non-human animals genetically engineered to contain one or more humanized Ig loci.

Preferably, the humanized antibodies of the present invention are prepared from transgenic non-human animals which generate antibody diversity primarily by gene conversion and hypermutation, e.g., rabbit, pigs, chicken, sheep, cow and horse. The antibodies can be made by immunizing transgenic animals with a desired antigen such as an infectious agent (e.g., bacteria or viruses) or parts or fragments thereof.

Such humanized antibodies have reduced immunogenicity to primates, especially humans, as compared to non-humanized antibodies prepared from non-human animals. Therefore, the humanized antibodies of the present invention are appropriate for use in the therapeutic treatment of human subjects.

Another embodiment of the present invention provides a preparation of humanized antibodies which can be monoclonal antibodies or polyclonal antibodies. Preferred antibody preparations of the present invention are polyclonal antibody preparations which, according to the present invention, have minimal immunogenicity to primates, especially humans.

A preferred preparation of polyclonal antibodies is composed of humanized immunoglobulin molecules having at least a heavy chain or light chain constant region polypeptide sequence encoded by a human constant region gene segment. More preferably, the variable domains of the heavy chains or light chains of the immunoglobulins molecules are also encoded by human gene segments.

In another embodiment, the present invention provides pharmaceutical compositions which include a preparation of humanized antibodies, and a pharmaceutically-acceptable carrier.

Another embodiment of the present invention provides novel sequences from the 5' and 3' flanking regions of the Ig gene segments of non-human animals, preferably, animals which rely primarily on gene conversion in generating the antibody diversity. In particular, the present invention provides novel nucleotide sequences downstream (3', 3-prime) of the genes coding for Cλ in chickens, Cγ and Cκ in rabbits, Cγ1,2,3 in cows and Cγ1,2 in sheep, as well as novel sequences 5' of rabbit Cγ.

In another embodiment, the present invention provides recombination vectors useful for replacing an Ig gene segment of a non-human animal with the corresponding human Ig gene segment. These vectors include a human Ig gene segment which is linked to flanking sequences at the 5' end and the 3' end, wherein the flanking sequences are homologous to the flanking sequences of the target animal Ig gene segment.

Preferred recombination vectors are those useful for the replacement of the animal's Ig constant region. For example, recombination vectors useful for replacing the rabbit heavy chain constant region genes are provided. A preferred vector contains from 5' to 3', a nucleotide sequence as set forth in SEQ ID NO: 12 or SEQ ID NO: 13, or a portion of SEQ ID NO: 12 or SEQ ID NO: 13, a human heavy chain constant region gene segment, a nucleotide sequence as set forth in SEQ ID NO: 10 or a portion of or SEQ ID NO: 10. Another preferred vector contains a nucleotide sequence as set forth in SEQ ID NO: 51, which sequence is characterized as having a human Cγ1 gene linked to flanking sequences from the 5' and 3' flanking regions of a rabbit heavy chain constant region gene.

Recombination vectors are also provided useful for replacing the rabbit light chain constant region genes. A preferred vector contains a nucleotide sequence as set forth in SEQ ID NO: 53, which sequence is characterized as having a human Cκ linked to flanking sequences from the 5' and 3' flanking regions of the rabbit light chain Cκ1 gene.

Other recombination vectors are provided which are useful for replacing the chicken light chain constant region genes. A preferred vector contains a nucleotide

sequence as set forth in SEQ ID NO: 57 which is characterized as having a human Cλ2 linked to flanking sequences from the 5' and 3' flanking regions of the chicken light chain Cλ gene.

Other recombination vectors provided include those useful for replacing the animal's Ig V region elements. For example, a recombination vector useful for replacing a rabbit heavy chain V region element is provided and contains SEQ ID NO: 52. A recombination vector useful for replacing a rabbit light chain V region element is provided and contains SEQ ID NO: 54.

In still another embodiment, the present invention provides transgenic constructs or vectors containing at least one humanized Ig locus, i.e., an Ig locus from a non-human animal or a portion of an Ig locus from a non-human animal wherein the locus or the portion of a locus is genetically modified to contain at least one human Ig gene segment. Such humanized Ig locus has the capacity to undergo gene rearrangement and gene conversion in the non-human animal thereby producing a diversified repertoire of humanized immunoglobulins.

One humanized Ig locus provided by the invention is a humanized heavy chain locus which includes one or more V gene segments, one or more D gene segments, one or more J gene segments, and one or more constant region gene segments, wherein at least one gene segment is a human heavy chain gene segment. The gene segments in the humanized heavy chain locus are juxtaposed with respect to each other in an unrearranged, or partially or fully rearranged configuration. A preferred humanized heavy chain locus contains a human constant region gene segment, preferably, Cα or Cγ. A more preferred humanized locus contains multiple V gene segments and at least one human V gene segment, in addition to a human heavy chain constant region segment. The human V gene segment is placed downstream of the non-human V gene segments.

Another humanized Ig locus is a humanized light chain locus which includes one or more V gene segments, one or more J gene segments, and one or more constant region gene segments, wherein at least one gene segment is a human light chain gene segment. The gene segments in the humanized light chain locus are juxtaposed with respect to each other in an unrearranged or rearranged configuration. A preferred

humanized light chain locus contains a human constant region gene segment, preferably, Cλ or Cκ. More preferably, the humanized light chain locus further contains multiple V gene segments and at least one human V gene segment. The human V gene segment is placed downstream of the non-human V gene segments. Even more preferably, the humanized light chain locus includes a rearranged human VJ segment, placed downstream of a number of (e.g., 10-100) VL gene segments of either non-human or human origin.

Another embodiment of the present invention is directed to methods of making a transgenic vector containing a humanized Ig locus by isolating an Ig locus or a portion of an Ig locus from a non-human animal, and integrating the desired human Ig gene segment(s) into the isolated animal Ig locus or the isolated portion of an Ig locus. The human Ig gene segment(s) are integrated into the isolated animal Ig locus or the isolated portion of an Ig locus by ligation or homologous recombination in such a way as to retain the capacity of the locus for undergoing effective gene rearrangement and gene conversion in the non-human animal. Integration of a human Ig gene segment by homologous recombination can be accomplished by using the recombination vectors of the present invention.

In another embodiment, the present invention provides methods of making transgenic animals capable of producing humanized antibodies. The transgenic animals can be made by introducing a transgenic vector containing a humanized Ig locus, or a recombination vector containing a human Ig gene segment, into a recipient cell or cells of an animal, and deriving an animal from the genetically modified recipient cell or cells.

Transgenic animals containing one or more humanized Ig loci, and cells derived from such transgenic animals (such as B cells from an immunized transgenic animal) are also provided. The transgenic animals of the present invention are capable of gene rearranging and gene converting the transgenic humanized Ig loci to produce a diversified repertoire of humanized immunoglobulin molecules.

Brief Description of the Drawings

Figure 1. Cow Cγ 3' flanking sequences. Primers are shown in shaded boxes. The 5' primer is in CH3, and the 3' primer is in M1. The sequences of clone 11, clone 3,

and clone 5 are set forth in SEQ ID NO: 3, SEQ ID NO: 4 and SEQ ID NO: 5, respectively.

Figure 2. Sheep Cγ 3' flanking sequences. Primers are shown in shaded boxes. The 5' primer is in CH3, and the 3' primer is in M2. The sequences of clone 11 and clone 1 are set forth in SEQ ID NO: 8 and SEQ ID NO: 9, respectively.

Figure 3. A novel 3' flanking sequence (SEQ ID NO: 10) of the rabbit Cgamma gene.

Figure 4. A novel nucleotide sequence (SEQ ID NO: 11) 3' of the rabbit Ckappa 1 gene.

Figure 5. Novel nucleotide sequences (SEQ ID NO: 12 and SEQ ID NO: 13) 5' of the rabbit Cgamma gene. The sequences between SEQ ID NO: 12 and SEQ ID NO: 13 (a gap of about 1000 nt) remain to be determined.

Figure 6. Comparison of human, mouse, rabbit, sheep, cow and camel sequences for the M1 and M2 regions 3' of the Cgamma gene.

Figure 7a. DNA construct for the replacement of rabbit Cκ with human Cκ. A 0.5 kb fragment containing a DNA sequence encoding human Ck is flanked by sequences from the rabbit Cκ1 gene. The upstream sequence (5'Cκ) is 2.8 kb, the downstream sequence (3'Cκ) is 2.6 kb. The vector also contains a lox-neo cassette for positive selection and a Hsv-Tk casette for negative selection.

Figure 7b. DNA construct for the replacement of rabbit Cγ with human Cγ1. A 1.8 kb fragment containing a DNA sequence encoding human Cγ1 is flanked by sequences from the rabbit Cγ gene. The upstream sequence (5'Cγ) is 1.9 kb, the downstream sequence (3'Cγ) is 3.1 kb. The vector also contains a lox-neo casette for positive selection and a Hsv-Tk cassette for negative selection. The figure is not up to scale.

Figure 8. DNA fragment (SEQ ID NO: 51) containing a human immunoglobulin heavy chain Cγ1 gene segment flanked by 50 nucleotides derived from the flanking regions of rabbit Cγ gene. Flanking sequences derived from the flanking regions of rabbit Cγ gene are underlined.

Figure 9. DNA fragment (SEQ ID NO: 52) containing a V gene segment with more than 80% sequence identity with rabbit V elements and encoding a human V element polypeptide sequence. Flanking sequences derived from the flanking regions of rabbit VH1 and J genes are underlined.

Figure 10. DNA fragment (SEQ ID NO: 53) containing a human immunoglobulin heavy chain Cκ gene segment flanked by 50 nucleotides derived from the rabbit light chain immunoglobulin Kappa1 gene. Flanking sequences derived from the flanking regions of rabbit Cκ gene are underlined.

Figure 11. DNA fragment (SEQ ID NO: 54) containing a V gene segment with more than 80% sequence identity with rabbit V elements and encoding a human V element polypeptide sequence. Flanking sequences derived from the flanking regions of rabbit immunoglobulin V and J genes are underlined.

Figure 12. DNA fragment (SEQ ID NO: 57) containing a gene encoding human immunoglobulin light chain constant region Clambda2 flanked by 50 nucleotides (underlined) derived from the flanking sequences of chicken Clambda gene.

Figure 13. Modification of the chicken light chain locus using the ET system. A chicken genomic BAC clone with the full-length light chain locus was modified by homologous recombination. In a first step Cλ was deleted by insertion of a selection cassette which was in a second homologous recombination step exchanged against the human Cλ gene.

Figure 14. DNA fragment (SEQ ID NO: 58) containing a VJ gene segment with 80% sequence identity with chicken V gene segments and encoding a human VJ immunoglobulin polypeptide. Flanking sequences derived from the flanking regions of chicken immunolgobulin V and J genes are underlined.

Figure 15. Modified chicken light chain locus.

Detailed Description of the Invention

One embodiment of the present invention provides humanized immunoglobulins (antibodies).

By "a humanized antibody" or "a humanized immunoglobulin" is meant an immunoglobulin molecule having at least a portion of a human immunoglobulin polypeptide sequence (or a polypeptide sequence encoded by a human Ig gene segment). The humanized immunoglobulin molecules of the present invention can be isolated from a transgenic non-human animal engineered to produce humanized immunoglobulin molecules. Such humanized immunoglobulin molecules are less immunogenic to primates, especially humans, relative to non-humanized immunoglobulin molecules prepared from the animal or prepared from cells derived from the animal.

The term "non-human animals" as used herein includes, but is not limited to, rabbits, pigs, birds (e.g., chickens, turkeys, ducks, geese and the like), sheep, goats, cows and horses. Preferred non-human animals are those animals which rely primarily on gene conversion and/or somatic hypermutation to generate antibody diversity, e.g., rabbit, pigs, birds (e.g., chicken, turkey, duck, goose and the like), sheep, goat, and cow. Particularly preferred non-human animals are rabbit and chicken.

In animals such as human and mouse, there are multiple copies of V, D and J gene segments on the heavy chain locus, and multiple copies of V and J gene segments on a light chain locus. Antibody diversity in these animals is generated primarily by gene rearrangement, i.e., different combinations of gene segments to form rearranged heavy chain variable region and light chain variable region. In other animals (e.g., rabbit, chicken, sheep, goat, and cow), however, gene rearrangement does not play a significant role in the generation of antibody diversity. For example, in rabbit, only a very limited number of the V gene segments, most often the V gene segments at the 3' end of the V-region, are used in gene rearrangement to form a contiguous VDJ segment. In chicken, only one V gene segment (the one adjacent to the D region, or "the 3' proximal V gene segment"), one D segment and one J segment are used in the heavy chain rearrangement; and only one V gene segment (the 3' proximal V segment) and one J segment are used in the light chain rearrangement. Thus, in these animals, there is little diversity among initially rearranged variable region sequences resulting from junctional diversification. Further diversification of the rearranged Ig genes is achieved by gene conversion, a

process in which short sequences derived from the upstream V gene segments replace short sequences within the V gene segment in the rearranged Ig gene.

The term “Ig gene segment” as used herein refers to segments of DNA encoding various portions of an Ig molecule, which are present in the germline of animals and humans, and which are brought together in B cells to form rearranged Ig genes. Thus, Ig gene segments as used herein include V gene segments, D gene segments, J gene segments and C region gene segments.

The term “human Ig gene segment” as used herein includes both naturally occurring sequences of a human Ig gene segment, degenerate forms of naturally occurring sequences of a human Ig gene segment, as well as synthetic sequences that encode a polypeptide sequence substantially identical to the polypeptide encoded by a naturally occurring sequence of a human Ig gene segment. By “substantially” is meant that the degree of amino acid sequence identity is at least about 85%-95%.

A preferred humanized immunoglobulin molecule of the present invention contains at least a portion of a human heavy or light chain constant region polypeptide sequence. A more preferred immunoglobulin molecule contains at least a portion of a human heavy or light chain constant region polypeptide sequence, and at least a portion of a human variable domain polypeptide sequence.

In another embodiment of the present invention, a preparation of humanized antibodies is provided.

By “a preparation of humanized antibodies” or “a humanized antibody preparation” is meant an isolated antibody product or a purified antibody product prepared from a transgenic non-human animal (e.g., serum, milk, or egg yolk of the animal) or from cells derived from a transgenic non-human animal (e.g., a B-cell or a hybridoma cell).

A humanized antibody preparation can be a preparation of polyclonal antibodies, which includes a repertoire of humanized immunoglobulin molecules. A humanized antibody preparation can also be a preparation of a monoclonal antibody.

Although the immunogenicity to humans of a humanized monoclonal antibody preparation is also reduced as compared to a non-humanized monoclonal antibody preparation, humanized polyclonal antibody preparations are preferred embodiments of

the present invention. It has been recognized that humanized monoclonal antibodies still invoke some degree of an immune response (an anti-idiotype response) in primates (e.g., humans) when administered repeatedly in large quantities because of the unique and novel idiotype of the monoclonal antibody. The present inventors have uniquely recognized that the overall immunogenicity of polyclonal antibodies is less dependent on an anti-idiotype response. For example, polyclonal antibodies made from non-human animals with only the constant region elements humanized (e.g., polyclonal antibodies having constant regions encoded by human gene segments, and having variable domains encoded by the endogenous genes of the non-human animal), are substantially non-immunogenic to primates.

Without intending to be bound to any theory, the present inventors have proposed that the reduced immunogenicity of such a humanized polyclonal antibody preparation is due to the fact that the preparation contains a very large number of different antibodies with many different idiotypes which are to a large extent defined by novel amino acid sequences in the complimentarity determining regions (CDR) of the heavy and light chain. Therefore, upon administration of such preparation into a primate such as a human, the administered amount of each individual immunoglobulin molecule in the preparation may be too low to solicit immune response against each immunoglobulin molecule. Thus, the humanized polyclonal antibody preparation which has many different idiotypes and variable regions has minimal immunogenicity to a recipient, even if the antibodies in the polyclonal antibody preparation are all directed to the same antigen. To further reduce any potential residual immunogenicity, a humanized polyclonal antibody preparation may be prepared which is composed of immunoglobulin molecules having both the variable domains and the constant regions encoded by human Ig gene segments.

In a preferred embodiment, the present invention provides an antibody preparation which includes humanized immunoglobulin molecules having at least a portion of a human heavy or light chain constant region polypeptide sequence. More preferably, the humanized immunoglobulines in the antibody preparation of the present invention further contain at least a portion of a human variable domain polypeptide

sequence, in addition to at least a portion of a human constant region polypeptide sequence.

Preferred humanized antibody preparations of the present invention are composed of humanized antibodies made from transgenic non-human animals whose antibody diversity is generated primarily by gene conversion, such as rabbit, birds (e.g., chicken, turkey, duck, goose and the like), sheep, goat, and cow; preferably, rabbit and chicken.

Once a transgenic non-human animal capable of producing diversified humanized immunoglobulin molecules is made (as further set forth below), humanized immunoglobulins and humanized antibody preparations against an antigen can be readily obtained by immunizing the animal with the antigen. A variety of antigens can be used to immunize a transgenic host animal. Such antigens include, microorganism, e.g. viruses and unicellular organisms (such as bacteria and fungi), alive, attenuated or dead, fragments of the microorganisms, or antigenic molecules isolated from the microorganisms.

Preferred bacterial antigens for use in immunizing an animal include purified antigens from *Staphylococcus aureus* such as capsular polysaccharides type 5 and 8, recombinant versions of virulence factors such as alpha-toxin, adhesin binding proteins, collagen binding proteins, and fibronectin binding proteins. Preferred bacterial antigens also include an attenuated version of *S. aureus*, *Pseudomonas aeruginosa*, enterococcus, enterobacter, and *Klebsiella pneumoniae,* or culture supernatant from these bacteria cells. Other bacterial antigens which can be used in immunization include purified lipopolysaccharide (LPS), capsular antigens, capsular polysaccharides and/or recombinant versions of the outer membrane proteins, fibronectin binding proteins, endotoxin, and exotoxin from Pseudomonas aeruginosa, enterococcus, enterobacter, and Klebsiella pneumoniae.

Preferred antigens for the generation of antibodies against fungi include attenuated version of fungi or outer membrane proteins thereof, which fungi include, but are not limited to, Candida albicans, Candida parapsilosis, Candida tropicalis, and Cryptococcus neoformans.

Preferred antigens for use in immunization in order to generate antibodies against viruses include the envelop proteins and attenuated versions of viruses which include, but are not limited to respiratory synctial virus (RSV) (particularly the F-Protein), Hepatitis C virus (HCV), Hepatits B virus (HBV), cytomegalovirus (CMV), EBV, and HSV.

Therapeutic antibodies can be generated for the treatment of cancer by immunizing transgenic animals with isolated tumor cells or tumor cell lines; tumor-associated antigens which include, but are not limited to, Her-2-neu antigen (antibodies against which are useful for the treatment of breast cancer); CD20, CD22 and CD53 antigens (antibodies against which are useful for the treatment of B cell lymphomas), (3) prostate specific membrane antigen (PMSA) (antibodies against which are useful for the treatment of prostate cancer), and 17-1A molecule (antibodies against which are useful for the treatment of colon cancer).

The antigens can be administered to a transgenic host animal in any convenient manner, with or without an adjuvant, and can be administered in accordance with a predetermined schedule.

After immunization, serum or milk from the immunized transgenic animals can be fractionated for the purification of pharmaceutical grade polyclonal antibodies specific for the antigen. In the case of transgenic birds, antibodies can also be made by fractionating egg yolks. A concentrated, purified immunoglobulin fraction may be obtained by chromatography (affinity, ionic exchange, gel filtration, etc.), selective precipitation with salts such as ammonium sulfate, organic solvents such as ethanol, or polymers such as polyethyleneglycol.

For making a monoclonal antibody, spleen cells are isolated from the immunized transgenic animal and used either in cell fusion with transformed cell lines for the production of hybridomas, or cDNAs encoding antibodies are cloned by standard molecular biology techniques and expressed in transfected cells. The procedures for making monoclonal antibodies are well established in the art. See, e.g., European Patent Application 0 583 980 A1 ("Method For Generating Monoclonal Antibodies From Rabbits"), U.S. Patent No. 4,977,081 ("Stable Rabbit-Mouse Hybridomas And Secretion

Products Thereof"), WO 97/16537 ("Stable Chicken B-cell Line And Method of Use Thereof"), and EP 0 491 057 B1 ("Hybridoma Which Produces Avian Specific Immunoglobulin G"), the disclosures of which are incorporated herein by reference. In vitro production of monoclonal antibodies from cloned cDNA molecules has been described by Andris-Widhopf et al., "Methods for the generation of chicken monoclonal antibody fragments by phage display", *J Immunol Methods 242:159* (2000), and by Burton, D. R., "Phage display", *Immunotechnology 1:87* (1995), the disclosures of which are incorporated herein by reference.

In a further embodiment of the present invention, purified monoclonal or polyclonal antibodies are admixed with an appropriate pharmaceutical carrier suitable for administration in primates especially humans, to provide pharmaceutical compositions. Pharmaceutically acceptable carriers which can be employed in the present pharmaceutical compositions can be any and all solvents, dispersion media, isotonic agents and the like. Except insofar as any conventional media, agent, diluent or carrier is detrimental to the recipient or to the therapeutic effectiveness of the antibodies contained therein, its use in the pharmaceutical compositions of the present invention is appropriate. The carrier can be liquid, semi-solid, e.g. pastes, or solid carriers. Examples of carriers include oils, water, saline solutions, alcohol, sugar, gel, lipids, liposomes, resins, porous matrices, binders, fillers, coatings, preservatives and the like, or combinations thereof

The present invention is further directed to novel nucleotide sequences and vectors, as well as the use of the sequences and vectors in making a transgenic non-human animal which produces humanized immunoglobulins.

In general, the genetic engineering of a non-human animal involves the integration of one or more human Ig gene segments into the animal's genome to create one or more humanized Ig loci. It should be recognized that, depending upon the approach used in the genetic modification, a human Ig gene segment can be integrated at the endogenous Ig locus of the animal (as a result of targeted insertion, for example), or at a different locus of the animal. In other words, a humanized Ig locus can reside at the chromosomal location where the endogenous Ig locus of the animal ordinarily resides, or at a chromosomal location other than where the endogenous Ig locus of the animal

ordinarily resides. Regardless of the chromosomal location, a humanized Ig locus of the present invention has the capacity to undergo gene rearrangement and gene conversion in the non-human animal thereby producing a diversified repertoire of humanized immunoglobulin molecules. An Ig locus having the capacity to undergo gene rearrangement and gene conversion is also referred to herein as a "functional" Ig locus, and the antibodies with a diversity generated by a functional Ig locus are also referred to herein as "functional" antibodies or a "functional" repertoire of antibodies.

In one embodiment, the present invention provides novel sequences useful for creating a humanized Ig locus and making transgenic animals capable of producing humanized immunoglobulin molecules. In particular, the present invention provides sequences from the 5' and 3' flanking regions of the Ig gene segments of non-human animals, preferably, animals which rely primarily on gene conversion in generating antibody diversity (e.g., rabbit, pigs, sheep, goat, cow, birds such as chicken, turkey, duck, goose, and the like).

The 5' and 3' flanking regions of the genes coding for the constant region are particularly important as these sequences contain untranslated regulatory elements (e.g., enhancers) critical for high Ig expression in the serum. The 3' flanking region of the genes coding for the constant region of the heavy chain also contain exons coding for the membranous and cytoplasmic tail of the membrane form of immunoglobulin (Volgina et al. *J Immunol* 165:6400, 2000). It has been previously established that the membrane and cytoplasmic tail of the membrane form of antibodies are critical in achieving a high level of expression of the antibodies in mice sera (Zou et al., *Science* 262:1271, 1993). Thus, the identification of the flanking sequences permits the replacement of exons and intervening introns of the Cγ gene with the human equivalent, and the maintenance of the endogenous exons encoding the transmembrane and cytoplasmic tail regions as well as the endogenous non-coding enhancer sequences.

In one embodiment, the present invention provides 3' flanking sequences of heavy chain constant regions of non-human animals. More particularly, nucleotide sequences downstream (3', 3-prime) of the genes coding for rabbit Cγ, cow Cγ1,2,3, and sheep Cγ1,2 are provided. Especially preferred nucleotide sequences include SEQ ID NO:

10 (3' of rabbit Cγ), SEQ ID NOS: 3-5 (3' of cow Cγ1,2,3), and SEQ ID NOS: 8-9 (3' of sheep Cγ1,2).

In another embodiment, the present invention provides 3' flanking sequences of light chain constant regions of non-human animals. More particularly, the present invention provides nucleotide sequences downstream (3', 3-prime) of the genes coding for Cκ in rabbits. Especially preferred nucleotide sequences include SEQ ID NO: 11 (3' of rabbit Cκ).

In still another embodiment, the present invention provides 5' flanking sequences of heavy chain constant regions of non-human animals. More particularly, nucleotide sequences upstream (5', 5-prime) of the rabbit Cγ gene are provided. Especially preferred sequences include SEQ ID NO: 12 and SEQ ID NO: 13.

Another embodiment of the present invention provides 5' flanking sequences of light chain constant regions of non-human animals.

Portions of the above novel flanking sequences are provided by the present invention. By "a portion" is meant a fragment of a flanking nucleotide sequence capable of mediating homologous recombination between the human Ig gene segment and the target animal Ig gene segment. Generally, a portion is at least about 200 base pairs, preferably, at least about 400 base pairs, for recombination in animal cells such as ES cells or fibroblasts, and at least about 40 base pairs, preferably at least about 50 base pairs, for recombination in *E. coli*. Examples of portions of the above novel flanking sequences include SEQ ID NOS: 59-60, 61-62, 63-64, 65-66, 67-68 and 69-70 (represented by the underlined sequences in Figures 8-12 and 14, respectively).

In a further aspect, the present invention provides vectors useful for the replacement of an Ig gene segment of a non-human animal with the corresponding human Ig gene segment. These vectors, also referred to herein as "recombination vectors", include a human Ig gene segment which is linked to flanking sequences at the 5' end and the 3' end, wherein the flanking sequences have a degree of homology with the flanking sequences of the target animal Ig gene segment sufficient to mediate homologous recombination between the human gene and the animal gene segments. Generally, at least about 200 bases should be identical between the flanking regions in a recombination

vector and the flanking regions of the target gene to achieve efficient homologous recombination in animal cells such as ES cells and fibroblasts; and at least about 40 bases should be identical to achieve efficient homologous recombination in *E. coli*.

Recombination vectors useful for replacing the animal's immunoglobulin heavy chain constant region genes are provided, which contain from 5' to 3', a nucleotide sequence homologous to the 5' flanking region of the target animal heavy chain constant region gene, a human heavy chain constant region gene (e.g., human $C\gamma1$), and a nucleotide sequence homologous to the 3' flanking region of the target animal heavy chain constant region gene.

Preferred recombination vectors are provided for the replacement of the rabbit heavy chain constant region genes. One such vector contains from 5' to 3', a nucleotide sequence as set forth in SEQ ID NO: 12 or SEQ ID NO: 13 or a portion thereof, a human heavy chain constant region gene segment, a nucleotide sequence as set forth in SEQ ID NO: 10 or a portion of or SEQ ID NO: 10. Another such vector contains SEQ ID NO: 51 (Figure 8) which is characterized as having a human $C\gamma1$ gene linked to flanking sequences from the 5' and 3' flanking regions of a rabbit heavy chain constant region gene.

Recombination vectors are also provided which are useful for replacing the animal's immunoglobulin light chain constant region genes. Such vectors contain from 5' to 3', a nucleotide sequence homologous to the 5' flanking region of the target light chain constant region gene, a human light chain constant region gene (e.g., human $C\kappa$ or $C\lambda$), and a nucleotide sequence homologous to the 3' flanking region of the target light chain constant region gene.

Preferred vectors include those useful for replacing the rabbit light chain constant region genes. A preferred vector contains a nucleotide sequence as set forth in SEQ ID NO: 53, which sequence is characterized as having a human $C\kappa$ linked to flanking sequences from the 5' and 3' flanking regions of the rabbit light chain $C\kappa1$ gene.

Other recombination vectors provided include those useful for replacing the animal's Ig V region elements. For example, a recombination vector useful for replacing a rabbit heavy chain V region element is provided and contains SEQ ID NO: 52. A

recombination vector useful for replacing a rabbit light chain V region element is provided and contains SEQ ID NO: 54.

The recombination vectors of the present invention can include additional sequences that facilitate the selection of cells which have undergone a successful recombination event. For example, marker genes coding for resistance to neomycin, bleomycin, puromycin and the like can be included in the recombination vectors to facilitate the selection of cells which have undergone a successful recombination event.

In a further aspect of the present invention, transgenic constructs or vectors carrying one or more humanized Ig loci are provided.

In one embodiment, the present invention provides transgenic constructs containing a humanized Ig heavy chain locus which includes one or more V gene segments, one or more D gene segments, one or more J gene segments, and one or more constant region gene segments, wherein at least one gene segment is a human heavy chain gene segment. The gene segments in such humanized heavy chain locus are juxtaposed wit respect to each other in an unrearranged configuration (or "the germline configuration"), or in a partially or fully rearranged configuration. The humanized heavy chain locus has the capacity to undergo gene rearrangement (if the gene segments are not fully rearranged) and gene conversion in the non-human animal thereby producing a diversified repertoire of heavy chains having human polypeptide sequences, or "humanized heavy chains".

In a preferred embodiment, the humanized heavy chain locus contains at least one C-region gene segment that is a human constant region gene segment, preferably, $C\alpha$ or $C\gamma$ (including any of the $C\gamma$ subclasses 1, 2, 3 and 4).

In another more preferred embodiment, the humanized heavy chain locus of the transgene contains a humanized V-region and a humanized C-region, i.e., a V-region having at least one human VH gene segment and a C-region having at least one human C gene segment (e.g., human $C\alpha$ or $C\gamma$).

Preferably, the humanized V-region includes at least about 10-100 heavy chain V (or "VH") gene segments, at least one of which is a human VH gene segment. In accordance with the present invention, the human VH gene segment included in the

transgene shares at least about 75% to about 85% homology to the VH gene segments of the host animal, particularly those animal VH gene segments included in the upstream region of the transgene. As described above, a human VH segment encompasses naturally occurring sequences of a human VH gene segment, degenerate forms of naturally occurring sequences of a human VH gene segment, as well as synthetic sequences that encode a polypeptide sequence substantially (i.e., at least about 85%-95%) identical to a human heavy chain V domain polypeptide.

Preferably, the human VH gene segment(s) is placed downstream of the non-human VH segments in the transgene locus. Preferably, the non-human VH gene segments in the transgene are the VH gene segments from the 3' VH-region in the Ig locus of the host animal, including the 3' proximal VH1.

In another embodiment, the present invention provides transgenic constructs containing a humanized light chain locus capable of undergoing gene rearrangement and gene conversion in the host animal thereby producing a diversified repertoire of light chains having human polypeptide sequences, or "humanized light chains".

The humanized light locus includes one or more V gene segments, one or more J gene segments, and one or more constant region gene segments, wherein at least one gene segment is a human light chain gene segment. The gene segments in the humanized light chain locus are juxtaposed in an unrearranged configuration (or "the germline configuration"), or fully rearranged configuration.

In a preferred embodiment, the humanized light chain locus contains at least one C-region gene segment that is a human constant region gene segment, preferably, Cλ or Cκ.

In another preferred embodiment, the humanized light chain locus of the transgene contains a humanized V-region and a humanized C-region, e.g., a V-region having at least one human VL gene and/or at least one rearranged human VJ segment, and a C-region having at least one human C gene segment (e.g., human Cλ or Cκ).

Preferably, the humanized V-region includes at least about 10-100 light chain V (or "VL") gene segments, at least one of which is a human VL gene segment. The human VL gene segment included in the transgene shares at least about 75% to about 85%

homology to the VL gene segments of the host animal, particularly those animal VL gene segments included in the upstream region of the transgene. Consistently, a human VL segment encompasses naturally occurring sequences of a human VL gene segment, degenerate forms of naturally occurring sequences of a human VL gene segment, as well as synthetic sequences that encode a polypeptide sequence substantially (i.e., at least about 85%-95%) identical to a human light chain V domain polypeptide.

Preferably, the human VL gene segment(s) is placed downstream of the non-human VL segments in the transgene locus. The non-human VL gene segments in the transgene construct are selected from the VL gene segments in the 3'VL-region in the light chain locus of the host animal, including the 3' proximal VL1.

In still another preferred embodiment, the humanized light chain locus includes a rearranged human VJ segment, placed downstream of a number of (e.g., 10-100) VL gene segments of either non-human or human origin.

Another aspect of the present invention is directed to methods of making a transgenic vector containing a humanized Ig locus. Such methods involve isolating an Ig locus or a portion thereof from a non-human animal, and inserting the desired human Ig gene segment(s) into the isolated animal Ig locus or the isolated portion of an animal Ig locus. The human Ig gene segment(s) are inserted into the isolated animal Ig locus or a portion thereof by ligation or homologous recombination in such a way as to retain the capacity of the locus of undergoing effective gene rearrangement and gene conversion in the non-human animal.

Preferably, DNA fragments containing an Ig locus to be humanized are isolated from animals which generate antibody diversity by gene conversion, e.g., rabbit and chicken. Such large DNA fragments can be isolated by screening a library of plasmids, cosmids, YACs or BACs, and the like, prepared from the genomic DNA of the non-human animal. An entire animal C-region can be contained in one plasmid or cosmid clone which is subsequently subjected to humanization. YAC clones can carry DNA fragments of up to 2 megabases, thus an entire animal heavy chain locus or a large portion thereof can be isolated in one YAC clone, or reconstructed to be contained in one YAC clone. BAC clones are capable of carrying DNA fragments of smaller sizes (about 150-

250 kb). However, multiple BAC clones containing overlapping fragments of an Ig locus can be separately humanized and subsequently injected together into an animal recipient cell, wherein the overlapping fragments recombine in the recipient animal cell to generate a continuous Ig locus.

Human Ig gene segments can be integrated into the Ig locus on a vector (e.g., a BAC clone) by a variety of methods, including ligation of DNA fragments, or insertion of DNA fragments by homologous recombination. Integration of the human Ig gene segments is done in such a way that the human Ig gene segment is operably linked to the host animal sequence in the transgene to produce a functional humanized Ig locus, i.e., an Ig locus capable of gene rearrangement and gene conversion which lead to the production of a diversified repertoire of humanized antibodies.

Preferably, human Ig gene segments are integrated into the Ig locus by homologous recombination. Homologous recombination can be performed in bacteria, yeast and other cells with a high frequency of homologous recombination events. For example, a yeast cell is transformed with a YAC containing an animal's Ig locus or a large portion thereof. Subsequently, such yeast cell is further transformed with a recombination vector as described hereinabove, which carries a human Ig gene segment linked to a 5' flanking sequence and a 3' flanking sequence. The 5' and the 3' flanking sequences in the recombination vector are homologous to those flanking sequences of the animal Ig gene segment on the YAC. As a result of a homologous recombination, the animal Ig gene segment on the YAC is replaced with the human Ig gene segment. Alternatively, a bacterial cell such as *E. coli* is transformed with a BAC containing an animal's Ig locus or a large portion thereof. Such bacterial cell is further transformed with a recombination vector which carries a human Ig gene segment linked to a 5' flanking sequence and a 3' flanking sequence. The 5' and the 3' flanking sequences in the recombination vector mediate homologous recombination and exchange between the human Ig gene segment on the recombination vector and the animal Ig gene segment on the BAC. Humanized YACs and BACs can be readily isolated from the cells and used in making transgenic animals.

In a further aspect of the present invention, methods of making transgenic animals capable of producing humanized immunoglobulins are provided.

According to the present invention, a transgenic animal capable of making humanized immunoglobulins are made by introducing into a recipient cell or cells of an animal one or more of the transgenic vectors described herein above which carry a humanized Ig locus, and deriving an animal from the genetically modified recipient cell or cells.

Preferably, the recipient cells are from non-human animals which generate antibody diversity by gene conversion and hypermutation, e.g., bird (such as chicken), rabbit, cows and the like. In such animals, the 3'proximal V gene segment is preferentially used for the production of immunoglobulins. Integration of a human V gene segment into the Ig locus on the transgene vector, either by replacing the 3'proximal V gene segment of the animal or by being placed in close proximity of the 3'proximal V gene segment, results in expression of human V region polypeptide sequences in the majority of immunoglobulins. Alternatively, a rearranged human V(D)J segment may be inserted into the J locus of the immunoglobulin locus on the transgene vector.

The transgenic vectors containing a humanized Ig locus is introduced into the recipient cell or cells and then integrated into the genome of the recipient cell or cells by random integration or by targeted integration.

For random integration, a transgenic vector containing a humanized Ig locus can be introduced into an animal recipient cell by standard transgenic technology. For example, a transgenic vector can be directly injected into the pronucleus of a fertilized oocyte. A transgenic vector can also be introduced by co-incubation of sperm with the transgenic vector before fertilization of the oocyte. Transgenic animals can be developed from fertilized oocytes. Another way to introduce a transgenic vector is by transfecting embryonic stem cells and subsequently injecting the genetically modified embryonic stem cells into developing embryos. Alternatively, a transgenic vector (naked or in combination with facilitating reagents) can be directly injected into a developing embryo. Ultimately, chimeric transgenic animals are produced from the embryos which contain the humanized Ig transgene integrated in the genome of at least some somatic cells of the transgenic animal.

In a preferred embodiment, a transgene containing a humanized Ig locus is randomly integrated into the genome of recipient cells (such as fertilized oocyte or developing embryos) derived from animal strains with an impaired expression of endogenous immunoglobulin genes. The use of such animal strains permits preferential expression of immunoglobulin molecules from the humanized transgenic Ig locus. Examples for such animals include the Alicia and Basilea rabbit strains, as well as Agammaglobinemic chicken strain. Alternatively, transgenic animals with humanized immunoglobulin transgenes or loci can be mated with animal strains with impaired expression of endogenous immunoglobulins. Offspring homozygous for an impaired endogenous Ig locus and a humanized transgenic Ig locus can be obtained.

For targeted integration, a transgenic vector can be introduced into appropriate animal recipient cells such as embryonic stem cells or already differentiated somatic cells. Afterwards, cells in which the transgene has integrated into the animal genome and has replaced the corresponding endogenous Ig locus by homologous recombination can be selected by standard methods. The selected cells may then be fused with enucleated nuclear transfer unit cells, e.g. oocytes or embryonic stem cells, cells which are totipotent and capable of forming a functional neonate. Fusion is performed in accordance with conventional techniques which are well established. See, for example, Cibelli et al., Science (1998) 280:1256. Enucleation of oocytes and nuclear transfer can also be performed by microsurgery using injection pipettes. (See, for example, Wakayama et al., Nature (1998) 394:369.) The resulting egg cells are then cultivated in an appropriate medium, and transferred into synchronized recipients for generating transgenic animals. Alternatively, the selected genetically modified cells can be injected into developing embryos which are subsequently developed into chimeric animals.

Further to the present invention, a transgenic animal capable of producing humanized immunoglobulins can also be made by introducing into a recipient cell or cells, one or more of the recombination vectors described herein above, which carry a human Ig gene segment, linked to 5' and 3' flanking sequences that are homologous to the flanking sequences of the endogenous Ig gene segment, selecting cells in which the endogenous Ig

gene segment is replaced by the human Ig gene segment by homologous recombination, and deriving an animal from the selected genetically modified recipient cell or cells.

Similar to the target insertion of a transgenic vector, cells appropriate for use as recipient cells in this approach include embryonic stem cells or already differentiated somatic cells. A recombination vector carrying a human Ig gene segment can be introduced into such recipient cells by any feasible means, e.g., transfection. Afterwards, cells in which the human Ig gene segment has replaced the corresponding endogenous Ig gene segment by homologous recombination, can be selected by standard methods. These genetically modified cells can serve as nuclei donor cells in a nuclear transfer procedure for cloning a transgenic animal. Alternatively, the selected genetically modified embryonic stem cells can be injected into developing embryos which can be subsequently developed into chimeric animals.

Transgenic animals produced by any of the foregoing methods form another embodiment of the present invention. The transgenic animals have at least one, i.e., one or more, humanized Ig loci in the genome, from which a functional repertoire of humanized antibodies is produced.

In a preferred embodiment, the present invention provides transgenic rabbits having one or more humanized Ig loci in the genome. The transgenic rabbits of the present invention are capable of rearranging and gene converting the humanized Ig loci, and expressing a functional repertoire of humanized antibodies.

In another preferred embodiment, the present invention provides transgenic chickens having one or more humanized Ig loci in the genome. The transgenic chickens of the present invention are capable of rearranging and gene converting the humanized Ig loci, and expressing a functional repertoire of humanized antibodies.

Cells derived from the transgenic animals of the present invention, such as B cells or cell lines established from a transgenic animal immunized against an antigen, are also part of the present invention.

In a further aspect of the present invention, methods are provided for treating a disease in a primate, in particular, a human subject, by administering a purified humanized

antibody composition, preferably, a humanized polyclonal antibody composition, desirable for treating such disease.

The humanized polyclonal antibody compositions used for administration are generally characterized by containing a polyclonal antibody population, having immunoglobulin concentrations from 0.1 to 100 mg/ml, more usually from 1 to 10 mg/ml. The antibody composition may contain immunoglobulins of various isotypes. Alternatively, the antibody composition may contain antibodies of only one isotype, or a number of selected isotypes.

In most instances the antibody composition consists of unmodified immunoglobulins, i.e., humanized antibodies prepared from the animal without additional modification, e.g., by chemicals or enzymes. Alternatively, the immunoglobulin fraction may be subject to treatment such as enzymatic digestion (e.g. with pepsin, papain, plasmin, glycosidases, nucleases, etc.), heating, etc, and/or further fractionated.

The antibody compositions generally are administered into the vascular system, conveniently intravenously by injection or infusion via a catheter implanted into an appropriate vein. The antibody composition is administered at an appropriate rate, generally ranging from about 10 minutes to about 24 hours, more commonly from about 30 minutes to about 6 hours, in accordance with the rate at which the liquid can be accepted by the patient. Administration of the effective dosage may occur in a single infusion or in a series of infusions. Repeated infusions may be administered once a day, once a week once a month, or once every three months, depending on the half-life of the antibody preparation and the clinical indication. For applications on epithelial surfaces the antibody compositions are applied to the surface in need of treatment in an amount sufficient to provide the intended end result, and can be repeated as needed.

The antibody compositions can be used to bind and neutralize antigenic entities in human body tissues that cause disease or that elicit undesired or abnormal immune responses. An "antigenic entity" is herein defined to encompass any soluble or cell-surface bound molecules including proteins, as well as cells or infectious disease-causing organisms or agents that are at least capable of binding to an antibody and preferably are also capable of stimulating an immune response.

Administration of an antibody composition against an infectious agent as a monotherapy or in combination with chemotherapy results in elimination of infectious particles. A single administration of antibodies decreases the number of infectious particles generally 10 to 100 fold, more commonly more than 1000-fold. Similarly, antibody therapy in patients with a malignant disease employed as a monotherapy or in combination with chemotherapy reduces the number of malignant cells generally 10 to 100 fold, or more than 1000-fold. Therapy may be repeated over an extended amount of time to assure the complete elimination of infectious particles, malignant cells, etc. In some instances, therapy with antibody preparations will be continued for extended periods of time in the absence of detectable amounts of infectious particles or undesirable cells. Similarly, the use of antibody therapy for the modulation of immune responses may consist of single or multiple administrations of therapeutic antibodies. Therapy may be continued for extended periods of time in the absence of any disease symptoms.

The subject treatment may be employed in conjunction with chemotherapy at dosages sufficient to inhibit infectious disease or malignancies. In autoimmune disease patients or transplant recipients, antibody therapy may be employed in conjunction with immunosuppressive therapy at dosages sufficient to inhibit immune reactions.

The invention is further illustrated, but by no means limited, by the following examples.

Example 1

Novel Sequences 3'prime of the Cγ Gene from Cows, Sheep and Rabbits

Genomic DNA was isolated from blood of a Simmental cow using the QIAamp DNA Blood Kit (QIAGEN). The genomic region 3' of the cow Cγ gene (i.e., the cow Cγ gene 3' flanking sequence) was PCR-amplified using the isolated genomic DNA as template and the following primers:

5' primer: 5'cgcaagcttCCTACACGTGTGTGGTGATG3' (SEQ ID NO: 1);

3' primer: 5'cgcaagcttAAGATGGWGATGGTSGTCCA3' (SEQ ID NO: 2)(Universal degenerate code: W=(A/T) S=(G/C)).

The upper-case portion of the 5' primer was from exon 3 of Cγ, and the lower-case portion represented a terminal HindIII restriction site. The upper-case portion of the 3' primer was a degenerate sequence designed according to the published sequences from the human M1 exon and the mouse M1 exon, and the lower-case portion represented a terminal HindIII restriction site. A 1.3kb PCR fragment was obtained using the EXPAND long template PCR system (Roche). The fragment was gel purified, digested with HindIII, and cloned into a Bluescript cloning vector. The resulting clones fell into three populations, which differ from one another in the pattern of the restriction fragments obtained with BamHI, EcoRI and XhoI. One clone from each population was sequenced, and the sequences are shown in Figure 1 (SEQ ID NOS: 3-5).

Genomic DNA was isolated from blood of a Merino sheep using the QIAamp DNA Blood Kit (QIAGEN). The genomic region 3' of the sheep Cγ gene (i.e., the sheep Cγ gene 3' flanking sequence) was PCR-amplified using the isolated genomic DNA as template and the following primers:

5' primer: 5'cgcggatccCCTACGCGTGTGTGGTGATG3' (SEQ ID NO: 6)

3' primer: 5'cgcggatccACCGAGGAGAAGATCCACTT3' (SEQ ID NO: 7)

The upper-case portion of the 5' primer was from exon 3 of Cγ, and the lower-case portion represented a terminal BamHI restriction site. The upper-case portion of the 3' primer was designed according to the published sequences from the human M2 exon and the mouse M2 exon, and the lower-case portion represented a terminal BamHI restriction site. A 2.9kb PCR fragment was obtained using the EXPAND long template PCR system (Roche). The fragment was gel purified, digested with BamHI, and cloned into a Bluescript cloning vector. The resulting clones fell into two populations, which differ from each other in the pattern of the restriction fragments obtained with HindIII, EcoRI and XhoI. One clone from each population was sequenced, and the sequences are shown in Figure 2 (SEQ ID NOS: 8-9).

A 10kb EcoRI fragment containing the Cγ gene and its flanking sequences from A2 allotype rabbit was subcloned from a genomic cosmid clone (cos 8.3 from

Knight et al., *J Immunol* (1985) 1245-50, "Organization and polymorphism of rabbit immunoglobulin heavy chain genes"). The nucleotide sequences 5' and 3' of Cγ were determined using standard methods and are set forth in Figure 3 and 5, SEQ ID NO: 10, 12, 13, respectively.

Sequences 3′ of rabbit Ckappa1 were determined from an EcoRI/BamHI subclone from VJk2Ck In pSV2neo. The nucleotide sequence is set forth in Figure 4, SEQ ID NO: 11.

The amino acid sequences encoded by the M1 and M2 exons from cow, sheep and rabbit were deduced from the above 3' flanking sequence. These amino acid sequences were aligned with the published M1 and M2 sequences from camel, human and mouse, as shown in Figure 6.

Example 2

A Vector for Replacing the Rabbit Endogenous Cγ Gene Segment with the Human Cγ1 Segment

Genomic DNA is isolated from rabbit fetal fibroblasts of an a2-homozygous rabbit. The DNA sequence upstream of rabbit Cγ (i.e., the 5' flanking sequence of rabbit Cγ) is amplified by PCR using the following primers:

5′ taattatgcggccgcCTTCAGCGTGAACCACGCCCTC 3′ (SEQ ID NO: 39) with a 5′ NotI site and

5′ GTCGACGCCCCTCGATGCACTCCCAGAG 3′ (SEQ ID NO: 40).

The DNA sequence downstream of rabbit Cγ (i.e., the 3' flanking sequence of rabbit Cγ) is amplified with the following primers:

5′ ggtaccCTCTCCCTCCCCCACGCCGCAGC 3′ (SEQ ID NO: 41) with a 5′ KpnI site and

5′ atatctcagaACTGGCTGTCCCTGCTGTAGTACACGG 3′ (SEQ ID NO: 42) with a 5′ XhoI site.

Human genomic DNA is isolated from human peripheral blood lymphocytes. The DNA fragment encoding human Cγ1 is amplified using the following primers:

5′ GTCGACACTGGACGCTGAACCTCGCGG 3′ (SEQ ID NO: 43) and

5′ GGTACCGGGGGCTTGCCGGCCGTCGCAC 3′ (SEQ ID NO: 44).

The fragments are digested with restriction enzymes and cloned into a Bluescript vector. Subsequently, a lox neo-cassette is inserted into the SalI site and an Hsv-tk cassette into the XhoI site. A schematic drawing of the final construct is shown in Figure 7a.

Example 3

A Vector for Replacing the Rabbit Endogenous Cκ Gene Segment with the Human Cκ Segment

Genomic DNA was isolated from rabbit fetal fibroblasts of a b5-homozygous rabbit. The DNA sequence upstream of rabbit Cκ1 (i.e., the 5' flanking sequence of rabbit Cκ1) was amplified by PCR using the following primers:

5′ gcggccgcTGGCGAGGAGACCAAGCTGGAGATCAAACG 3′ (SEQ ID NO: 45) with a 5′ NotI site

5′ GTCGACGCAGCCCAAAGCTGTTGCAATGGGGCAGCG 3′ (SEQ ID NO: 46).

The DNA sequence downstream of rabbit Cκ1 (i.e., the 5' flanking sequence of rabbit Cκ1) was amplified with the following primers:

5′ atatggtaccGCGAGACGCCTGCCAGGGCACCGCC 3′ (SEQ ID NO: 47) with a 5′ KpnI site

5′ GGATCCCGAGCTTTATGGGCAGGGTGGGGG 3′ (SEQ ID NO: 48).

Human genomic DNA was isolated from human peripheral blood lymphocytes. The DNA fragment encoding human Cκ was amplified using the following primers:

5′ ATATGTCGACCTGGGATAAGCATGCTGTTTTCTGTCTGTCCC 3′ (SEQ ID NO: 49)

5′ CTAGGTACCAGCAGGTGGGGGCACTTCTCCC 3′ (SEQ ID NO: 50).

The fragments were digested with restriction enzymes and cloned into a Bluescript vector. Subsequently, a lox neo-cassette was inserted into the SalI site and an Hsv-tk cassette into the XhoI site. A schematic drawing of the final construct is shown in Figure 7b.

Example 4

Replacement of the Endogenous Cγ and Cκ Gene Segments in Rabbit Fetal Fibroblasts with the Corresponding Human Gene Segments

Rabbit fetal fibroblast cells are prepared by standard methods. After one passage, fibroblasts are transfected with 5μg of the NotI-linearized targeting vector as shown in Figure 5a for Cγ or Figure 51b for Cκ, and are seeded in 96-well plates (2 x 10^3 cells/well). After a positive selection with 600μg/ml G418 and a negative selection with 200nM FIAU, resistant colonies are replica-plated to two 96-well plates for DNA analysis and cryopreservation, respectively. PCR and/or Southern blot analysis is performed to identify cells with the human Cγ1 gene segment integrated in the genome. The cells having the integrated human Cγ1 gene are used in rabbit cloning as described in Example 5.

Example 5

Cloning of Rabbits

Mature Dutch Belton rabbits are superovulated by subcutaneous injection of follicle stimulating hormone (FSH) every 12 hours (0.3 mg x 2 and 0.4 mg x 4). Ovulation is induced by intravenous administration of 0.5 mg luteinizing hormone (LH) 12 hours after the last FSH injection. Oocytes are recovered by ovidual flush 17 hours after LH injection. Oocytes are mechanically enucleated 16-19 hours after maturation. Chromosome removal is assessed with bisBENZIMIDE (HOECHST 33342, Sigma, St. Louis, MO) dye under ultraviolet light. Enucleated oocytes are fused with actively dividing fibroblasts by using one electrical pulse of 180 V/cm for 15 us (Electrocell Manipulator 200, Genetronics, San Diego, CA). After 3-5 hours oocytes are chemically activated with calcium ionophore (6 uM) for 4 min (# 407952, Calbiochem, San Diego, CA) and 2 mM 6-dimethylaminopurine (DMAP, Sigma) in CR2 medium (Specialty Media, Lavalett, NJ) with 3 mg/ml bovine serum albumin (fatty acid free, Sigma) for 3 hours. Following the activation, the embryos are washed in hamster embryo culture

medium (HECM)-Hepes five times and subsequently, cultivated in CR2 medium containing 3 mg/ml fatty-acid free BSA for 2-48 hours at 37.8° C and 5%CO_2 in air. Embryos are then transferred into synchronized recipients. Offsprings are analyzed by PCR for a segment of the transgene.

Example 6

Construction of a DNA Fragment Containing a Portion of a Rabbit Heavy Chain Locus with a Human Cγ1 Gene Segment and a VH Gene Segment Encoding a Human VH Domain Polypeptide Sequence

The upstream and downstream regions (i.e., the 5' and 3' flanking regions) of the rabbit heavy chain Cγ gene from an a2-allotype rabbit were sequenced. A DNA fragment (SEQ ID NO: 51) is generated by PCR using overlapping oligonucleotides wherein the DNA fragment contains from 5' to 3', a sequence derived from the 5' flanking region of the rabbit Cγ gene, the human Cγ1 gene, and a sequence derived from the 3' flanking region of the rabbit Cγ gene (Figure 8).

A genomic BAC library derived from an a2-allotype rabbit is generated by standard procedures and screened with probes specific for rabbit Cγ. A BAC clone containing rabbit heavy chain gene segments is identified. The rabbit Cγ gene on this BAC clone is replaced with the human Cγ1 gene by homologous recombination in *E.coli* using the DNA fragment of SEQ ID NO: 51 and the pET system. This replacement is accomplished by two consecutive recombination steps: first the rabbit Cγ gene segment is replaced with a marker gene; then the marker gene is replaced the human Cγ1 gene segment.

The modified BAC clone containing rabbit heavy chain genes and the inserted human Cγ1 gene is further modified by replacing the 3'proximal VH1 segment with a synthetic VH gene segment (Figure 9). This synthetic VH gene segment (SEQ ID NO: 52) is made using overlapping oligonculeotides and includes a 5' flanking sequence, a 3' flanking sequence, and a sequence coding for a polypeptide nearly identical to the human immunoglobulin heavy chain variable domain polypeptide sequence described by Huang

and Stollar (*J. Immunol.* 151: 5290-5300, 1993). The coding sequence of the synthetic VH gene segment is designed based on the published sequence of a rabbit VH1 gene (a2, Knight and Becker, *Cell* 60:963-970, 1990) and is more than 80% identical to rabbit VH gene segments. The 5' and the 3' flanking sequences in the synthetic VH segment are derived from the upstream and downstream regions of the a2-allotype rabbit VH1 gene. The synthetic VH gene of SEQ ID NO: 52 is used to replace the rabbit VH1 gene on the BAC clone by homologous recombination using the pET or the redεβγ system. The modified BAC clone is amplified and purified using standard procedures.

Example 7

Construction of a DNA Fragment Containing a Portion of a Rabbit Light Chain Locus with a Human Cκ Gene Segment and a VJ Gene Segment Encoding a Human VL Domain Polypeptide Sequence

The upstream and downstream regions (i.e., the 5' and 3' flanking regions) of the rabbit light chain Cκ1 gene from a b5-allotype rabbit were sequenced. A DNA fragment (SEQ ID NO: 53) is generated by PCR using overlapping oligonucleotides wherein the DNA fragment contains from 5' to 3', a sequence derived from the 5' flanking region of the rabbit Cκ1 gene, the human Cκ1 gene, and a sequence derived from the 3' flanking region of the rabbit Cκ1 gene (Figure 10).

A genomic BAC library derived from a b5-allotype rabbit is generated by standard procedures and screened with probes specific for rabbit Cκ1. A BAC clone containing rabbit light chain gene segments is identified. The rabbit Cκ1 gene on this BAC clone is replaced with the human Cκ1 gene on the DNA fragment of SEQ ID NO: 53 by homologous recombination in *E.coli* using the pET or the redεβγ system. This replacement is accomplished by two consecutive recombination steps: first the rabbit Cκ1 gene segment is replaced with a marker gene; then the marker gene is replaced the human Cκ1 gene segment.

The modified BAC clone containing rabbit light chain genes and the inserted human Cκ1 gene is further modified by inserting a rearranged VJ DNA fragment into the

J region of the rabbit light chain locus. The rearranged VJ DNA fragment encodes a human immunoglobulin variable domain polypeptide described by Pritsch et al. (*Blood* 82(10):3103-3112, 1993) and Lautner-Rieske et al. (*Eur. J. Immunol.* 22 (4), 1023-1029, 1992)) (Figure 7). The nucleotide sequence of the rearranged VJ fragment is designed to maximize the sequence homology at the nucleotide level to the rabbit Vkappa sequence published by Lieberman et al. (*J. Immunol.* 133 (5), 2753-2756, 1984). This rearranged VJ DNA sequence is more than 80% identical with known rabbit Vκ genes. Using overlapping oligonucleotides in PCR, the rearranged VJ DNA fragment is linked to a 5' and a 3' flanking sequence, resulting the DNA fragment of SEQ ID NO: 54 (Figure 11). The 5'flanking sequence is derived from 5' of a rabbit Vκ, the 3'flanking sequence is derived from 3' of rabbit J2. The DNA fragment of SEQ ID NO: 54 is subsequently inserted into the rabbit light chain locus by homologous recombination in *E. coli* using the pET or the redεβγ system. The insertion is performed in such a way that the rabbit light chain region containing the rabbit Vκ1 gene segment, the rabbit J1 and J2 segments, and the sequences in between, is replaced with the rearranged VJ DNA fragment. Again, this insertion is accomplished by replacement of the rabbit V to J region with a marker gene, followed by the replacement of the marker gene with the rearranged VJ DNA fragment. The modified BAC clone is amplified and purified using standard procedures.

Example 8

Transgenic Rabbits Expressing the Humanized Immunoglobulin Light and/or Heavy Chain Transgene

Transgenic rabbits are generated as described by Fan et al. (*Pathol. Int.* 49: 583-594, 1999). Briefly, female rabbits are superovulated using standard methods and mated with male rabbits. Pronuclear-stage zygotes are collected from oviduct and placed in an appropriate medium such as Dulbecco's phosphate buffered saline supplemented with 20% fetal bovine serum. The exogenous DNA (e.g., the humanized BAC clone from Example 4 and/or 5 which has been linearized prior to injection) is microinjected into the male pronucleus with the aid of a pair of manipulators. Morphological surviving zygotes

are transferred to the oviducts of pseudopregnant rabbits. Pseudopregnancy is induced by the injection of human chorionic gonadotrophin (hCG). Between about 0.1-1% of the injected zygotes develop into live transgenic rabbits. Integration of the transgene in the genome is confirmed by Southern blots analysis using a probe specific for the transgene.

cDNA is prepared using RNA isolated from B cells (in blood, spleen and/or lymph nodes) of a transgenic rabbit. Primers specific for the human transgene (human CH gene segment or the synthetic humanized VH gene segment) are used to generate amplified products from cDNA. The observation of amplified products indicates that the transgene is rearranged in the transgenic animal and the rearranged transgene is transcribed in the animal. Amplified products are sequenced and the presence of donor sequences from upstream V genes indicates that the transgene introduced into the germline of the animal undergoes gene conversion.

The presence of antibodies containing human IgG and/or human kappa light chain antigenic determinants in the serum of transgenic founder rabbits is determined using an ELISA assay.

Example 9

Production of Humanized Antibodies From Transgenic Rabbits with the Genetic Background of the Alicia and/or Basilea Rabbit Strain

The Alicia strain lacks the VH1 gene segment and therefore has an impaired Ig heavy chain expression. Transgenic founder rabbits capable of expressing humanized heavy chain molecules in the genetic background of the Alicia rabbit strain are generated, e.g., by using fetal fibroblasts established from Alicia rabbits in Examples 4-5 above, or by using zygotes from female Alicia rabbits mated with male Alicia rabbits in Example 8 above. Transgenic animals are also obtained which are homozygous for the Alicia Ig phenotype and are also homozygous for a humanized heavy chain transgene. Serum is tested in ELISA for the presence of humanized heavy chain (e.g., a human heavy chain

constant region). The concentration of antibodies with humanized Ig heavy chains in these homozygous Alicia animals is substantially higher, e.g., about 10 to 100 fold higher, than that produced from a transgene integrated in the genome of wild type (non-Alicia) rabbits.

The Basilea strain does not express κ1 light chain and in its place exclusively express the κ2 and λ light chains. Transgenic founder rabbits capable of expressing humanized light chain molecules in the genetic background of the Basilea rabbit strain are generated, e.g., by using fetal fibroblasts established from Basilea rabbits in Examples 4-5 above, or by using zygotes from female Basilea rabbits mated with male Basilea rabbits in Example 8 above. Transgenic animals are obtained which are homozygous for the Basilea light chain phenotype, and are also homozygous for a humanized light chain transgene. Serum is tested in ELISA for the presence of the humanized light chain. The concentration of the humanized light chain in the homozygous Basilea animals is substantially higher, about 10-100 fold higher, than the concentration of a humanized light chain in a transgenic rabbit with the wild type (non-Basilea) genetic background. Transgenic founder rabbits are mated with each other to generate transgenic rabbits with the following traits: (1) having at least one humanized light chain transgene, (2) having at least one humanized heavy chain transgene, (3) homozygous for the Alicia heavy chain locus, and (4) homozygous for the Basilea light chain locus.

Example 10

Construction of a DNA Fragment Containing a Modified Chicken Light Chain Locus Having a Human Clambda2 Gene Segment and a VJ Gene Segment Encoding a Human VL Domain

A genomic BAC library derived from a jungle fowl chicken was screened with radiolabeled probes specific for chicken light chain Clambda and chicken Vpsi25 (the V gene segment at the very 5' end of the light chain locus). A BAC clone containing the entire lambda light chain locus was identified. The chicken Cλ gene on this BAC clone is replaced with the human Cλ2 gene by homologous recombination in *E.coli* using the pET system (Zhang et al., *Nat. Biotechnol.* 18(12):1314-7, 2000) as follows.

A first DNA fragment containing a kanamycin selection cassette was generated by PCR using primers specific for Tn5 gene. The 5' primer (5'catacacagccatacatacgcgtgtggccgctctgcctctctcttgcaggTATGGACAGCAAGCGAACCG 3', SEQ ID NO: 55) was designed to include 50 bp at the 5' end (lower case), derived from the 5' flanking region of the chicken light chain Cλ gene. The 3' primer (5'atcagggtgacccctacgttacactcctgtcaccaaggagtgggagggacTCAGAAGAACTCGTCAAGAAG3', SEQ ID NO: 56) was designed to include about 50 bp at the end (lower case), derived from the 3' flanking region of the chicken light chain Cλ gene.

A second DNA fragment (SEQ ID NO: 57) was synthesized using overlapping oligonucleotides wherein the DNA fragment contains from 5' to 3', a sequence derived from the 5' flanking region of the chicken light chain Clambda gene, the human Clambda2 gene, and a sequence derived from the 3' flanking region of the chicken Clambda gene (Figure 12).

E. coli cells of the chicken light chain BAC clone were transformed with a recombination plasmid expressing the recE and recT functions under an inducible promotor. Cells transformed with the recombination plasmid were then transformed with the first DNA fragment above and selected afterwards in media containing kanamycin. Clones resistant to kanamycin were identified, and the replacement of the chicken Cλ segment by the kanamycin selection cassette via homologous recombination was confirmed by restriction enzyme digest.

In the second homologous recombination step, cells positive for the kanamycin selection cassette were transformed with the second DNA fragment above. Transformed cells were screened for the loss of kanamycin resistance as indicative of the replacement of the kanamycin selection cassette by the human Cλ2 gene. The exchange was confirmed by restriction enzyme digest and/or sequence analysis.

The ET cloning procedure is summarized in Figure 13.

The BAC clone containing the chicken light chain locus and the inserted human Clambda2 gene segment was further modified by inserting a rearranged VJ DNA fragment. The rearranged VJ DNA fragment encodes a human immunoglobulin variable domain polypeptide described by Kametani et al. (*J. Biochem.* 93 (2), 421-429, 1983) as

IG LAMBDA CHAIN V-I REGION NIG-64 (P01702) (Figure 14). The nucleotide sequence of the rearranged VJ fragment was so designed as to maximize the sequence homology at the nucleotide level to the chicken Vlambda1 sequence published by McCormack et al. (*Cell* 56, 785-791, 1989). This rearranged VJ DNA sequence is more than 80% identical with known chicken light chain V genes. The rearranged VJ DNA fragment was linked to a 5' flanking sequence and a 3' flanking sequence, resulting in the DNA fragment of SEQ ID NO: 58 (Figure 14). The 5' flanking sequence was derived from 5' of chicken Vlambda1, and the 3'flanking sequence was derived from 3' of chicken J. The DNA fragment of SEQ ID NO: 58 was subsequently inserted into the chicken light chain locus in *E. coli* using the pET system as shown in Figure 15. The insertion was performed in such a way that the region on the chicken light chain locus from the 5' end of the chicken Vlambda1 gene segment to the 3' end of the chicken J region was replaced with the rearranged, synthetic VJ DNA fragment. Again, this insertion wais accomplished by the replacement of the chicken V-J region with a marker gene, followed by the replacement of the marker gene with the rearranged VJ DNA fragment. The modified region of the chicken light chain locus is shown in Figure 15. The modified BAC clone was amplified and purified using standard procedures.

Example 11

Construction of a DNA Fragment Containing a Portion of a Chicken Heavy Chain Locus With a Human Cγ1 Gene Segment and a VH Gene Segment Encoding a Human VH Domain Polypeptide Sequence

A jungle fowl chicken genomic BAC library was generated by standard procedures and screened with probes specific for chicken Cγ. A BAC clone containing chicken heavy chain gene segments is identified. The upstream and downstream regions (i.e., the 5' and 3' flanking regions) of the heavy chain Cγ gene are sequenced. The chicken Cγ gene on this BAC clone is replaced with the human Cγ1 gene by homologous recombination in *E.coli* using the pET system as follows.

A first DNA fragment containing a kanamycin selection cassette is generated by PCR using primers specific for Tn5 gene. The 5' and 3' primers are designed to

include about 50 bp at the end, derived from the 5' and 3' flanking regions of the chicken heavy chain Cγ gene.

A second DNA fragment is generated by PCR using overlapping oligonucleotides wherein this second DNA fragment contains from 5' to 3', a sequence of about 50 bp derived from the 5' flanking region of the chicken Cγ gene, the human Cγ1 gene, and a sequence of about 50 bp derived from the 3' flanking region of the chicken Cγ gene.

E. coli cells of the chicken CY BAC clone are transformed with a recombination plasmid expressing the recE and recT functions under an inducible promotor. Cells transformed with the recombination plasmid are further transformed with the first DNA fragment and selected in media containing kanamycin. Clones resistant to kanamycin are identified, and the replacement of the chicken CY segment by the kanamycin selection cassette via homologous recombination is confirmed by restriction enyme digest.

In the second homologous recombination step, cells positive for the kanamycin selection cassette are now transformed with the second DNA fragment described above. Transformed cells are screened for loss of kanamycin resistance as indicative of the replacement of the kanamycin selection cassette by the human Cγ1 gene. The exchange is confirmed by restriction enzyme digest and/or sequence analysis.

The BAC clone containing the inserted human Cγ1 gene is further modified by replacing the 3'proximal VH1 segment (i.e., the 3'proximal VH1 gene in the V region) with a synthetic VH gene segment. This synthetic VH gene segment is designed based on the published sequence of a chicken VH1 gene (Arakawa et al., EMBO J 15(10): 2540-2546, 1996). The synthetic gene segment is more than 80% identical to chicken VH gene segments and encodes an amino acid sequence that is identical to the amino acid sequence of a human immunoglobulin heavy chain variable domain polypeptide described by Matthyssens and Rabbitss (in Steinberg CM and Lefkovits I, (eds). *The Immune System*: 132-138, S. Karger, NY 1981). This synthetic VH segment including 5' and 3' flanking sequences is synthesized by PCR using overlapping oligonucleotides. The 5' and the 3' flanking sequences are derived from the upstream and downstream regions of chicken

VH1 gene. This synthetic VH segment is used to replace the chicken VH1 gene on the BAC clone by homologous recombination using the pET system. The modified BAC clone is amplified and purified using standard procedures.

Example 12

Transgenic Chicken Expressing the Humanized Immunoglobulin Light and/or Heavy Chain Transgenes

The production of transgenic chicken is carried out using techniques as described by Etches et al., *Methods in Molecular Biology* 62: 433-450; Pain et al., *Cells Tissues Organs* 1999; 165(3-4): 212-9; Sang, H., "Transgenic chickens--methods and potential applications", *Trends Biotechnol 12:415* (1994); and in WO 200075300, "Introducing a nucleic acid into an avian genome, useful for transfecting avian blastodermal cells for producing transgenic avian animals with the desired genes, by directly introducing the nucleic acid into the germinal disc of the egg".

Briefly, the modified BAC clones are linearized and mixed with a transfection reagent to promote uptake of DNA into cells. The formulations are injected into a multicell stage chicken embryo in close proximity to the germinal disc. The window in the egg shell is closed and the eggs are incubated. After hatching chimeric chickens are identified by PCR and Southern blot analysis using transgene specific sequences. Integration of the transgene in the genome is confirmed by Southern blots analysis using a probe specific for the transgene. Heavy and light chain transgenic animals are bred with each other to generate transgenic chickens expressing antibodies having humanized heavy and light chains.

cDNA is prepared using RNA isolated from B cells (in blood, spleen and/or lymph nodes) from transgenic chickens. Primers specific for the human transgene (e.g., human CH gene segments and/or the synthetic humanized VH gene segments) are used to generate amplified products from cDNA. The observation of amplified products indicates that the transgene is rearranged in the transgenic animal and the rearranged transgene is transcribed in the animal. Amplified products are sequenced and the presence of donor

sequences from upstream V genes indicates that the transgene introduced into the germline of the animal undergoes gene conversion.

The presence of antibodies containing human IgG and/or human kappa light chain antigenic determinants in the serum of transgenic chickens is determined using an ELISA assay.

Example 13

Production of Functional Humanized Antibodies *in Transgenic Chicken with the Agammaglobulinemic Phenotype*

Transgenic chickens with the following traits are produced: (1) having at least one humanized light chain transgene, (2) having at least one humanized heavy chain transgene, and (3) homozygous for the agammaglobulinemic phenotype. These animals produce antibodies into the blood and eggs, and antibodies can be purified from either source. In general, antibody concentrations in the eggs are about 5% to 50% of antibodies concentration in the blood. Animals that contain humanized antibodies at high levels in eggs can be selected and bred to produce offspring. Alternatively, transgenic animals can be generated that specifically secrete humanized antibodies into their eggs.

Example 14

Generation Of Transgenic Chickens Expressing Humanized Immunoglobulin

Chicken embryonic stem cells are isolated and cultured as described by Pain et al. (*Development* 122, 2339-2348; 1996). Chicken embryos are obtained from eggs immediately after they are laid. The entire blastoderm is removed by gentle aspiration, embryos are slowly dissociated mechanically and cells are seeded in ESA complete medium on inactivated STO feeder cells. ESA medium is composed of MEM medium containing 10% FCS, 2% chicken serum, 1% bovine serum albumin, 10 ng/ml ovalbumin, 1 mM sodium pyruvate, 1% non-essential amino acids, 1 μM of each nucleotide

adenosine, guanosine, cytidine, uridine, thymidine, 0.16 mM β-mercaptoethanol, ESA complete medium is supplemented with 10 ng/ml bFGF, 20 ng/ml h-IGF-1, 1% vol/vol avian-SCF and 1% vol/vol h-LIF, 1% vol/vol h-IL-11. Cell cultures are incubated wt 37°C in 7.5 CO_2 and 90% humidity. After 48 hours fresh blastodermal cells are added to the culture in half of the original volume of ESA complete medium. After an additional incubation for three days, the culture medium is partially (50%) replaced with fresh ESA complete medium, and totally every day thereafter. For cell harvesting, cultures are washed with PBS and incubated in a pronase solution (0.025% w/v). Dissociated cells are transfected with various linearized transgenic constructs containing a humanized Ig locus. Transfected cells are incubated with STO feeder cells (as described above) in the presence of selective antibiotics. Cells are transferred onto fresh feeder cells twice per week. Antibiotic resistant cells are isolated and the integration of a humanized Ig gene fragments at a random site or at the corresponding chicken immunoglobulin gene loci is confirmed by PCR.

Subsequently, genetically modified cells are injected into recipient embryos. As recipient embryos, freshly laid eggs are irradiated (6Gy - Cobalt source). Between 100 to 200 genetically modified cells are injected into the subgerminal cavity using a micropipet. The window in the egg shell is closed and the eggs are incubated. Somatic chimerism of hatched chickens is evaluated by PCR. Germ-line chimerism is assessed by mating of somatic chimeras.

Example 15
Immunization Of Transgenic Animals

Genetically engineered chickens are immunized intramuscularly with purified Hepatitis B surface antigen (HBsAg) (5μg in incomplete Freund's adjuvant) on day 0, 14 and day 28. On day 35 animals are bled and serum is prepared. ELISA plates (NUNC, Denmark) are coated with 1 μg/ml HBsAg in PBS for 1 hour at room temperature. Subsequently, available binding sites are blocked by incubation with 1% non-fat dry milk (NFM) in PBS (300 μl/well). Chicken serum is diluted in PBS/1%NFM and added to the

coated wells. After an incubation of 1 hour, the plates are washed 3 times with PBS/0.05% Tween 20 and bound Ig is detected using goat anti-human Ig conjugated with horseradish peroxidase. Conjugated goat antibody is detected using o-phenylenediamine dihydrochloride (Sigma) at 1 mg/ml. The colorimetric reaction is stopped by addition of 1 M HCl solution and the absorbance is measured at 490 nm. As a control, serum from non-immunized chicken is used. Serum from non-immunized chickens does not react with HBsAg. At a dilution of 1:250 the optical density measured in uncoated and HBsAg coated wells is below 0.2. In contrast, serum from immunized chickens contains humanized antibodies reactive with HBsAg. At a serum dilution of 1:250 the measured optical density is 2.3. Upon further dilution of the serum the measured optical density declines to 0.1 (at a dilution of 25600). No antibodies reactive with a goat anti-chicken IgG-HRP conjugate can be detected. This demonstrates that the genetically engineered chickens produce humanized anti-HBsAg antibodies following immunization.

Genetically engineered rabbits are immunized intramuscularly with purified Hepatitis B surface antigen (HBsAg) (10μg in incomplete Freund's adjuvant) on day 0 and day 14. On day 28 animals are bled from the ear and serum is prepared. ELISA plates (NUNC, Denmark) are coated with 1 μg/ml HBsAg in PBS for 1 hour at room temperature. Subsequently, available binding sites are blocked by incubation with 1% non-fat dry milk (NFM) in PBS (300 μl/well). Rabbit serum is diluted in PBS/1%NFM and added to the coated wells. After an incubation of 1 hour, the plates are washed 3 times with PBS/0.05% Tween 20 and bound Ig is detected using goat anti-human Ig conjugated with horse-radish peroxidase. Conjugated goat antibody is detected using o-phenylenediamine dihydrochloride (Sigma) at 1 mg/ml. The colorimetric reaction is stopped by addition of 1 M HCl solution and the absorbance is measured at 490 nm. As a control serum from non-immunized rabbits is used. Serum from non-immunized rabbits does not react with HBsAg. At a dilution of 1:100 the optical density measured in uncoated and HBsAg coated wells is below 0.4. In contrast, serum from immunized rabbits contains partially human antibodies reactive with HBsAg. At a serum dilution of 1:100 the measured optical density is 2.8. Upon further dilution of the serum the measured optical density declines to 0.2 (at a dilution of 25600). No antibodies reactive

with a goat anti-rabbit IgG-HRP conjugate can be detected. This demonstrates that the genetically engineered rabbits produce humanized anti-HBsAg antibodies following immunization.

Example 16

Complement Mediated Cytotoxicity of Virus Infected Cell Line Using Humanized Antibodies

A human liver carcinoma cell line expressing HBsAg is labeled with 0.1 mCi ^{51}Cr in 100 ul PBS for 1 hr at 37°C. Two thousand ^{51}Cr-lableled cells are incubated with serum from genetically engineered rabbits or chickens expressing anti-HbsAg humanized immunoglobulins. After two hours at 37°C the release of ^{51}Cr into the supernatant is determined by measuring radioactivity using a scintillation counter. For the determination of maximum release, 1% Triton X100 is added. The degree of cell lysis is calculated as follows: %Lysis = CPM experimental ±CPM#spontaneous / CPM# total ± CPM spontaneous. Incubation of labeled cells with serum (diluted 1:30) from non-immunized rabbits does not result in cell lysis (<10%). However, incubation of cells with serum from immunized rabbits causes 80% cell lysis. Inactivation of complement in the serum by heat treatment (56°C for 30 minutes) renders the serum from immunized rabbits inactive. These results demonstrate that humanized antibodies produced by genetically engineered rabbits bind to HBsAg-positive cells and cause complement dependent lysis.

Example 17

Immunization of Transgenic Animals against Staphylococcus aureus

Genetically engineered chickens are immunized intramuscularly with a recombinant fragment of the Staphylococcus aureus collagen adhesin protein (100µg in incomplete Freund's adjuvant) on day 0, 14 and day 28. On day 35 animals are bled and serum is prepared. ELISA plates (NUNC, Denmark) are coated with 2 µg/ml collagen adhesin protein in PBS for 1 hour at room temperature. Subsequently, available binding sites are blocked by incubation with 1% non-fat dry milk (NFM) in PBS (300 µl/well).

Chicken serum is diluted in PBS/1%NFM and added to the coated wells. After an incubation of 1 hour, the plates are washed 3 times with PBS/0.05% Tween 20 and bound Ig is detected using goat anti-human Ig conjugated with horseradish peroxidase. Conjugated goat antibody is detected using o-phenylenediamine dihydrochloride (Sigma) at 1 mg/ml. The colorimetric reaction is stopped by addition of 1 M HCl solution and the absorbance is measured at 490 nm. As a control, serum from non-immunized chicken is used. Serum from non-immunized chickens does not react with collagen adhesin protein. At a dilution of 1:250 the optical density measured in uncoated and collagen adhesin protein coated wells is below 0.2. In contrast, serum from immunized chickens contains humanized antibodies reactive with collagen adhesin. At a serum dilution of 1:250 the measured optical density is 2.3. Upon further dilution of the serum the measured optical density declines to 0.1 (at a dilution of 25600). No antibodies reactive with a goat anti-chicken IgG-HRP conjugate can be detected. This demonstrates that the genetically engineered chickens produce humanized anti-Staph. aureus collagen adhesin antibodies following immunization.

Genetically engineered rabbits are immunized intramuscularly with recombinant fragment of the Staphylococcus aureus collagen adhesin protein (100μg in incomplete Freund's adjuvant) on day 0 and day 14. On day 35 animals are bled and serum is prepared. ELISA plates (NUNC, Denmark) are coated with 2 μg/ml collagen adhesin protein in PBS for 1 hour at room temperature. Subsequently, available binding sites are blocked by incubation with 1% non-fat dry milk (NFM) in PBS (300 μl/well). Rabbit serum is diluted in PBS/1%NFM and added to the coated wells. After an incubation of 1 hour, the plates are washed 3 times with PBS/0.05% Tween 20 and bound Ig is detected using goat anti-human Ig conjugated with horseradish peroxidase. Conjugated goat antibody is detected using o-phenylenediamine dihydrochloride (Sigma) at 1 mg/ml. The colorimetric reaction is stopped by addition of 1 M HCl solution and the absorbance is measured at 490 nm. As a control, serum from non-immunized rabbit is used. Serum from non-immunized rabbits does not react with collagen adhesin protein. At a dilution of 1:250 the optical density measured in uncoated and collagen adhesin protein coated wells is below 0.2. In contrast, serum from immunized rabbits contains

humanized antibodies reactive with collagen adhesin. At a serum dilution of 1:250 the measured optical density is 2.3. Upon further dilution of the serum the measured optical density declines to 0.1 (at a dilution of 25600). No antibodies reactive with a goat anti-rabbit IgG-HRP conjugate can be detected. This demonstrates that the genetically engineered rabbits produce humanized anti-Staph. aureus collagen adhesin antibodies following immunization.

Example 18

Protection Against *Staphylococcus Aureus* Infection In A Mouse Model

Naive mice are passively immunized i.p. on day -1 with 16 mg of the immunoglobulin fraction containing antibodies specific for the *S. aureus* collagen adhesin protein (from Example 17) or with the immunoglobulin fraction from non-immunized animals. On day 0, the mice are challenged i.v. with $4x10^7$ CFU *S. aureus* per mouse and mortality is monitored over the next 7 days. Mortality rate in the control groups is 80% and 10% in the group treated with the immunoglobulin fraction containing antibodies specific for the *S. aureus* collagen adhesin protein. The data indicate that anticollagen adhesin antibodies can protect mice against lethal *S. aureus* challenge.

Example 19

Antigen-Specific Hybridomas Made From Transgenic Animals.

Transgenic animals are immunized with an antigen (e.g., KLH, human red blood cells or sheep red blood cells). Spleen cells are removed at various times after immunization and fused with myeloma cell lines derived from rabbit and chicken, respectively. After fusion cells are plated into 96 well plates and supernatants are tested for the presence of humanized antibodies. To demonstrate that the antibodies contain human immunoglobulin sequences, hybridomas are stained with fluorescent-labeled antibodies reactive with human heavy and light chain immunoglobulins. Limiting dilution is conducted to purify hybridomas to monoclonality.

Example 20

Evaluation of Immunogenicity

Serum samples are collected from five cynomologous monkeys on day 0. Subsequently, a purified partially human polyclonal antibody preparation (5 mg/kg) is administered into five cynomologous monkeys by intravenous administration. The administration is repeated six times in bi-weekly intervals. Monkeys are monitored closely for any side-effects (e.g., anaphylactic shock, reflected by an elevated body temperature). After seven months serum is collected from blood samples. Affinity resins containing purified human IgG or partially human IgG are produced by standard procedure using CNBr-activated Sepharose. Monkey serum samples (3 ml) are added to the IgG-affinity resin (4 ml) containing 10 mg human or partially human IgG. Subsequently, the columns are washed with PBS. Bound monkey immunoglobulin is eluted from the column with 0.1M glcyin/HCl pH2.5 and dialyzed 2 times against PBS. The protein content of the eluted fractions is determined using the BCA assay using human IgG as a standard. The total amounts of protein in these fractions demonstrate that therapy with partially human IgG does not lead to a significant antibody response in the treated animals.

Example 21

Treating Animals Using Humanized Antibodies

Humanized polyclonal immunoglobulins are purified from the serum of genetically engineered rabbits, or from egg yolk of genetically engineered chickens, by ammonium sulfate precipitation and ion exchange chromatography. SCID-mice are injected with one million human liver carcinoma cells expressing HBsAg. Subsequently, 25 μg immunoglobulin is injected peritoneally once per day. Animals treated with antibodies isolated from non-immunized rabbit serum die after about 60 days. This is similar to untreated recipients of liver carcinoma cells. In contrast, mice treated with antibodies isolated from immunized rabbit serum survive for more than 150 days. This

demonstrates that human antibodies produced in genetically engineered rabbits are capable of eliminating human carcinoma cells from SCID-mice.

What is claimed is:

1. An isolated nucleic acid molecule comprising the sequence as set forth in any one of SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, or SEQ ID NO: 13, or a portion of any one of SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, or SEQ ID NO: 13.

2. A recombination vector for replacing an Ig gene segment from a non-human animal with a human Ig gene segment, comprising from 5' to 3', a 5' nucleotide sequence, said human Ig gene segment, and a 3' nucleotide sequence, wherein said 5' nucleotide sequence and said 3' nucleotide sequence are homologous to the 5' and 3' flanking sequences of said Ig gene segment from the non-human animal.

3. The recombination vector of claim 2, wherein said non-human animal is an animal which relies primarily on gene conversion in generating antibody diversity.

4. The recombination vector of claim 3, wherein said animal is rabbit, pig, chicken, sheep or cow.

5. The recombination vector of claim 3, wherein the Ig gene segment from a non-human animal is a gene segment coding for a heavy chain or light chain constant region.

6. The recombination vector of claim 5, wherein said vector comprises from 5' to 3', a 5' nucleotide sequence as set forth in any one of SEQ ID NO: 12, SEQ ID NO: 13, a portion of SEQ ID NO: 12, or a portion of SEQ ID NO: 13; a human heavy chain constant region gene segment; a 3' nucleotide sequence as set forth in SEQ

ID NO: 10 or a portion of or SEQ ID NO: 10; and wherein said vector is useful for replacing a rabbit heavy chain constant region gene segment.

7. The recombination vector of claim 5, comprising the nucleotide sequence as set forth in SEQ ID NO: 51 wherein said vector is useful for replacing a rabbit heavy chain constant region gene segment.

8. The recombination vector of claim 5, wherein said vector is useful for replacing a rabbit light chain constant region gene and comprises a nucleotide sequence as set forth in SEQ ID NO: 53.

9. The recombination vector of claim 5, wherein said vector is useful for replacing a chicken light chain constant region gene and comprises a nucleotide sequence as set forth in SEQ ID NO: 57.

10. The recombination vector of claim 3, wherein the Ig gene segment from a non-human animal is a gene segment coding for a heavy chain or light chain variable region.

11. The recombination vector of claim 10, wherein said vector is useful for replacing a rabbit heavy chain variable region gene and comprises a nucleotide sequence as set forth in SEQ ID NO: 52.

12. The recombination vector of claim 10, wherein said vector is useful for replacing a rabbit light chain variable region gene and comprises a nucleotide sequence as set forth in SEQ ID NO: 54.

13. A transgenic vector comprising a humanized Ig locus, wherein said humanized Ig locus is derived from an Ig locus or a portion of an Ig locus of a non-human animal and comprises multiple Ig gene segments wherein at least one of said gene

segments is a human Ig gene segment, wherein said gene segments are juxtaposed in an unrearranged, partially rearranged or fully rearranged configuration, and wherein said humanized Ig locus is capable of undergoing gene conversion and producing a repertoire of humanized immunoglobulins in said non-human animal.

14. The transgenic vector of claim 13, wherein said non-human animal is an animal which generates antibody diversity substantially by gene conversion.

15. The transgenic vector of claim 14, wherein said non-human animal is rabbit, pig, chicken, sheep or cow.

16. The transgenic vector of claim 13, wherein said humanized Ig locus is a heavy chain locus and comprises at least one V gene segment, at least one D gene segment, at least one J gene segment and at least one constant region gene segment.

17. The transgenic vector of claim 16, wherein said constant region gene segment is a human heavy chain constant region gene segment.

18. The transgenic vector of claim 17, wherein said human heavy chain constant region gene segment is a Cγ.

19. The transgenic vector of claim 17, comprising about 10-100 V gene segments and at least one human V gene segment, wherein said human V gene segment is placed downstream to said 10-100 V gene segments.

20. The transgenic vector of claim 19, wherein said V gene segments are selected from V gene segments at the 3' V-region of said non-human animal and human V gene segments.

21. The transgenic vector of claim 13, wherein said humanized Ig locus is a light chain locus and comprises at least one V gene segment, at least one J gene segment and at least one constant region gene segment.

22. The transgenic vector of claim 21, wherein said constant region gene segment is a human light chain constant region gene segment.

23. The transgenic vector of claim 22, wherein said human light chain constant region gene segment is Cλ or Cκ.

24. The transgenic vector of claim 22, comprising about 10-100 V gene segments and at least one human V gene segment, wherein said human V gene segment is placed downstream to said 10-100 V gene segments.

25. The transgenic vector of claim 24, wherein said V gene segments are selected from V gene segments at the 3' V-region of said non-human animal and human V gene segments.

26. The transgenic vector of claim 22, wherein said human V gene segment is placed immediately 5' to a J gene segment in a rearranged configuration.

27. A method of making a transgenic vector comprising a humanized Ig locus capable of producing a functional repertoire of humanized antibodies in a non-human animal, comprising:

   (i) obtaining a DNA fragment comprising an Ig locus or a portion thereof from said non-human animal which comprises at least one V gene segment, at least one J gene segment and at least one constant region gene segment; and

   (ii) integrating at least one human Ig gene segment into said DNA fragment of step (i) to produce a humanized Ig locus, wherein said human Ig

gene segment is linked to the sequences of non-human origin operably as to permit gene rearrangement and gene conversion of said humanized Ig locus and the production of a functional repertoire of humanized antibodies in said non-human animal.

28. The method of claim 27, wherein the integration of said human Ig gene segment is achieved by homologous recombination, thereby replacing an Ig gene segment in said Ig locus or said portion thereof from said non-human animal.

29. The method of claim 28, wherein the homologous recombination is achieved in a bacterial cell, a yeast cell, or a non-human animal cell.

30. The method of claim 28, wherein the human Ig gene segment is provided on a recombination vector, and is linked to a 5' nucleotide sequence and a 3' nucleotide sequence which are homologous to the 5' and 3' flanking sequences of said Ig gene segment from the non-human animal.

31. A transgenic animal comprising a humanized Ig locus, wherein said humanized Ig locus is derived from an Ig locus or a portion of an Ig locus of a non-human animal and comprises multiple Ig gene segments wherein at least one of said gene segments is a human Ig gene segment, said gene segments being juxtaposed in an unrearranged, partially rearranged or fully rearranged configuration, and wherein said humanized Ig locus is capable of undergoing gene conversion and producing a repertoire of humanized immunoglobulins in said non-human animal.

32. The transgenic animal of claim 31, wherein said animal is selected from rabbit, pig, chicken, sheep or cow.

33. A B cell from the transgenic animal of claim 31.

34. A method of making a transgenic non-human animal capable of producing a functional repertoire of humanized Ig heavy chains, comprising:
   (i) introducing a transgenic construct according to any one of claims 16-20 into a recipient cell of a non-human animal and integrating the humanized heavy chain locus in the transgenic construct into the genome of said recipient cell; and
   (ii) deriving an animal from the recipient cell having the humanized heavy chain locus integrated in the genome, thereby producing a functional repertoire of humanized Ig heavy chains.

35. The method of claim 34, wherein said animal is rabbit and said recipient cell is a cell in an early embryo.

36. The method of claim 35, wherein said rabbit has an impaired expression of endogenous Ig molecules.

37. The method of claim 34, wherein said animal is chicken and said recipient cell is a fertilized egg.

38. The method of claim 37, wherein said chicken has an impaired expression of endogenous Ig molecules.

39. A method of making a transgenic non-human animal capable of producing a functional repertoire of humanized Ig light chains, comprising:
   (i) introducing a transgenic construct according to any one of claims 21-26 into a recipient cell of a non-human animal and integrating the humanized light chain locus in the transgenic construct into the genome of said non-human animal; and

(ii) deriving an animal from the recipient cell having the humanized light locus integrated in the genome, thereby producing a functional repertoire of humanized Ig light chains.

40. The method of claim 39, wherein said animal is rabbit and said recipient cell is a cell in an early embryo.

41. The method of claim 40, wherein said rabbit has an impaired expression of endogenous Ig molecules.

42. The method of claim 39, wherein said animal is chicken and said recipient cell is a fertilized egg.

43. The method of claim 42, wherein said chicken has an impaired expression of endogenous Ig molecules.

44. A method of making a transgenic non-human animal capable of producing a functional repertoire of humanized antibodies, comprising:

(i) introducing a transgenic construct according to any one of claims 16-20 and a transgenic construct according to any one of claims 21-26 into a recipient cell of a non-human animal, and integrating the humanized Ig loci in the transgenes into the genome of said non-human animal; and

(ii) deriving an animal from the recipient cell having the humanized Ig loci integrated in the genome, thereby producing a functional repertoire of humanized antibodies.

45. A method of making a transgenic non-human animal capable of producing a functional repertoire of humanized antibodies, comprising

(i) making a transgenic non-human animal capable of producing a functional repertoire of humanized heavy chains;

(ii) making a transgenic non-human animal capable of producing a functional repertoire of humanized light chains; and

(iii) mating the transgenic non-human animal of (i) with the transgenic animal of (ii); and

(iv) selecting an offspring which produces both humanized heavy chains and humanized light chains thereby obtaining a transgenic non-human animal capable of producing a functional repertoire of humanized antibodies.

46. A humanized immunoglobulin produced using the transgenic animal of claim 31.

47. A humanized immunoglobulin derived from a transgenic animal, comprising at least a portion of a human imunglobulin polypeptide sequence.

48. The humanized immunoglobulin of claim 47, wherein said transgenic animal generates antibody diversity by gene conversion and/or hypermutation

49. The humanized immunoglobulin of claim 48, wherein said transgenic animal is a rabbit, chicken, sheep or cow.

50. The humanized immunoglobulin of claim 49, wherein said human immunglobulin polypeptide sequence is a heavy chain or light chain polypeptide sequence.

51. The humanize immunoglobulin of claim 50, wherein said portion of a human immunglobulin polypeptide sequence is a human constant region polypeptide sequence.

52. The humanized immunoglobulin of claim 51, wherein said human constant region polypeptide sequence is Cγ, Cκ, or Cλ.

53. The humanized immunoglobulin of claim 51, wherein said portion of a human immunoglobulin polypeptide sequence further comprising a human V domain polypeptide sequence.

54. The humanized immunoglobulin of claim 47, wherein said humanized immunoglobulin is specific for an antigen.

55. The humanized immunoglobulin of claim 54, wherein said antigen is a microorganism selected from bacterium, fungus, or virus; an antigenic portion of said organism; an antigenic molecule derived from said microorganism; or a tumor-associated antigen.

56. The humanized immunoglobulin of claim 55, wherein said bacterim is selected from *S. aureus*, *Pseudomonas aeruginosa*, enterococcus, enterobacter, or *Klebsiella pneumoniae.*

57. The humanized immunoglobulin of claim 55, wherein said fungus is selected from Candida albicans, Candida parapsilosis, Candida tropicalis, or Cryptococcus neoformans.

58. The humanized immunoglobulin of claim 55, wherein said virus is selected from respiratory synctial virus (RSV), Hepatitis C virus (HCV), Hepatits B virus (HBV), cytomegalovirus (CMV), EBV, or HSV.

59. The humanized immunoglobulin of claim 55, wherein said antigen is selected from Her-2-neu antigen, CD20, CD22, CD53, prostate specific membrane antigen (PMSA), or17-1A molecule.

60. An antibody preparation, comprising the humanized immunoglobulin of any one of claims 46-55.

61. The antibody preparation of claim 60, wherein said preparation is a monoclonal antibody preparation.

62. The antibody preparation of claim 60, wherein said preparation is a polyclonal antibody preparation.

63. The antibody preparation of claim 62, wherein said preparation is substantially non-immunogenic to human.

64. A pharmaceutical composition, comprising a pharmaceutically acceptable carrier and the antibody preparation of claim 60.

65. A method of treating a disease in a human subject comprising administering to said subject a thereapeutically effective amount of the antibody preparation of claim 60.

66. The method of claim 59, wherein said disease is caused by bacterial, fungal or viral infection, or said disease is a cancer.

Figure 1(a)-(d). Novel nucleotide sequences 3'prime of the cow Cgamma gene (Cow Cγ 3′ flanking sequences). Primers are shown in shaded boxes. The 5′ primer is in CH3, and the 3′ primer is in M1. The sequences of clone 11, clone 3, and clone 5 are set forth in SEQ ID NO: 3, SEQ ID NO: 4 and SEQ ID NO: 5, respectively.

```
          1                                                 50
clone11   CCTAC ACGTGTGTGG TGATGCACGA AACTTTACGG
 clone3   CCTAC ACGTGTGTGG TGATGCACGA GGCCCTGCAC
 clone5   CCTAC ACGTGTGTGG TGATGCACGA GGCCCTGCAC

          151                                              200
clone11   AATCACTACA AAGAGAAGTC CACCTCGAGG TCTCCGGGTA AATGAGCCTC
 clone3   AATCACTACA CGCAGAAGTC CACCTCTAAG TCTGCGGGTA AATGAGCCTC
 clone5   AATCACTACA CGCAGAAGTC CACCTCTAAG TCTGCGGGTA AATGAGCCTC

          201                                              250
clone11   GCGCCGCTGA TCTAGTGGAC GTTCCCTCAT CCACCCACCC CTCCCCCCAC
 clone3   ACGTCCCTGC ACCAGCAAGC CCTCACCCAG C......... ..CCACCCTC
 clone5   ACGTCCCTGC ACCAGCAAGC CCTCACCCAG C......... ..CCACCCTC

          251                                              300
clone11   CCCGGGCTCC AGGTCCAGCC AGGGCGCCCT AGCCCCTCCC TGTGTGCATT
 clone3   CCCGGGCTCC AAGTCCAGCC AGGACGCCCT AGCCCCTCCC TGTGTGCATT
 clone5   CCCGGGCTCC AGGTCCAGCC AGGACGCCCT AGCCCCTCCC TGTGTGCATT

          301                                              350
clone11   CCTCCTGGGC CGCCGTGAAT AAAGCACCCA GGCCGCCCTG GGACCCTGCA
 clone3   CCTCCTGGGC CGCCGTGAAT AAAGCACCCA GGCCACCCTG GGACCCTGCA
 clone5   CCTCCTGGGC CGCCGTGAAT AAAGCACCCA GGCCGCCCTG GGACCCTGCA

          351                                              400
clone11   ACGCTGTGCT GGTTCTTTCC GAGGCAGAGC CCTGGTGGCC GCCAGGCCTG
 clone3   ACGCTGTGCT GGTTCTTTCC GAGGCAGAGC CCTGGTGGCC GCCAGGCCTG
 clone5   ACGCTGTGCT GGTTCTTTCC GAGGCAGAGC CCTGGTGGCC GCCAGGCCTG

          401                                              450
clone11   CGGGGGTGGG CTGAGCCGAC TCTGGGCCAC TTTGTTCAGC ATCTGTGGGG
 clone3   CAGGGGTGGG CTGAGCCGAC TCTGGGCCAC TTTGTTCAGC ATCTGTGGGG
 clone5   CGGGGGTGGG CTGAGCCGAC TCTGGGCCAC TTTGTTCAGC ATCTGTGGGG

          451                                              500
clone11   GAGCTGACCC CACTCCGGGC CAGACACACA GTGAGTGGGT CCAGCAGGCC
 clone3   GAGCTGACCC CGCTCCGGGC CAGACACACA GTGAGTGGGT CCAGCAGGCC
 clone5   GAGCTGACCC CACTCCGGGC CAGACACACA GTGAGTGGGT CCAGCAGGCC

          501                                              550
clone11   ACCTGGGGGC TGCCCAAGGC CACAGAGGGG CTTGGCCAGA GGCACAGCTC
 clone3   ACCTGGGGGC TGCCCGAGGC CACGGAGGGG CTTGGCCAGA GGCGTACCTC
 clone5   ACCTGGGGGC TGCCCAAGGC CACAGAGGGG CTTGGCCAGA GGCACAGCTC
```

Figure 1(a)

```
        551                                                600
clone11 CACGGTCCCC TCCAGCCACC ACCTGCTGGG CCGGCCTCTG GACAGGAACC
 clone3 CACGGCCCCC TCCAGCCACC ACCTGCTGGG CCGGCCTCTG GACAGGAACC
 clone5 CACGGTCCCC TCCAGCCACC ACCTGCTGGG CCGGCCTCTG GACAGGAACC

        601                                                650
clone11 GGGGAAGCCC CCGAGACCCT CAGGGATTGA GGCCCAATGC TTCCCGCCTC
 clone3 GGGGAAGCCC CCGAGACCCT CAGGGATTGA GGCCCAATGC TTCCCGCCTC
 clone5 GGGGAAGCCC CCGAGACCCT CAGGGATTGA GGCCCAATGC TTCCCGCCTC

        651                                                700
clone11 TGCTCCAGCC CACGCTGTGG GGCAGGGCCA CATCCTTGTC CCCAGGCCCC
 clone3 TGCTCCAGCC CACGCTGTGG GGCAGGGCCA CATCCTTGTC CCCAGGCCCC
 clone5 TGCTCCAGCC CACGCTGTGG GGCAGGGCCA CATCCTTGTC CCCAGGCCCC

        701                                                750
clone11 TGTCCTTGGG TGTCCAGAGT CCTTGTGTCC ACTCTGGGCC TGCCTGGAGC
 clone3 TGTCCCTGGG TGCCCAGAGT CCTTGTGTCC ACTCTGGGCC TGCCTGGAGC
 clone5 TGTCCTTGGG TGTCCAGAGT CCTTGTGTCC ACTCTGGGCC TGCCTGGAGC

        751                                                800
clone11 CACGCATGGC CAGGGGGTGG CCCTGCTTCA CCCTCAGGCT CCCAAGGTCA
 clone3 CACGCGTGGC CGGGGGATGG CCCTGCTCCA CCCTCAGGCT CCCAAGGTCA
 clone5 CACGCATGGC CAGGGGGTGG CCCTGCTTCA CCCTCAGGCT CCCAAGGTCA

        801                                                850
clone11 GGCCTCGCCC TCCCTCGGCC AGGAGGCTCT GCCCGGCTCT CCCTGCCCAG
 clone3 GGCCTCGCCC TCCCTCAGCC AGGAGGCTCT GCCCGGCTCT CCCTGCCCAG
 clone5 GGCCTCGCCC TCCCTCGGCC AGGAGGCTCT GCCCGGCTCT CCCTGCCCAG

        851                                                900
clone11 GGCCAGGCCT GTGCGCCCAT GGGGAGGTCA TCCCTGTGCC TGAAAGGGGT
 clone3 GGCCAGGCCT GTGCGCCCAT GGGGAGGTCA TCCCTGTGCC TGAAAGGGCT
 clone5 GGCCAGGCCT GTGCGCCCAT GGGGAGGTCA TCCCTGTGCC TGAAAGGGGT

        901                                                950
clone11 CCAGGCCGAG AGCCCTGAAT GTCCAGGGCA GGGACCTAGC TGCTCCCTGT
 clone3 CCAGGCCGGG AGCCCTGAAT GTCCAGGGCA GGGACCTAGC TGCTCCCTGC
 clone5 CCAGGCCGAG AGCCCTGAAT GTCCAGGGCA GGGACCTAGC TGCTCCCTGT

        951                                               1000
clone11 GGACACGGAG CCCAGAGCCA CAGACAACAA GCCCCAGCCC CGCACGCACA
 clone3 AGACACGGAG CCCAGAGCCA CAGACAACAA GCCCCAGCCC CGCACGCACA
 clone5 GGACACGGAG CCCAGAGCCA CAGACAACAA GCCCCAGCCC CGCACGCACA

        1001                                              1050
clone11 CGAGACAGCC CGCACCCAGC CTCCTCCACA CGCACTCAGG TGTACATGCG
 clone3 CAAGACAGCC CGCACCCAGC CTCCTCCACA CGCACTCAGG TGTGCATCCG
 clone5 CGAGACAGCC CACACCCCGC CTCCTCCACA CGCACTCAGG TGTGCATCCG
```

Figure 1(b)

```
        1051                                                  1100
clone11 CACATGAGCA CACTTCACCC CGTCACACCC ACACACCTAC ACACACTCAG
 clone3 CACATGAGCA CACTTCACCC CGTCACACCC ACACGCCTAC ACACACTCAG
 clone5 CACATGAGCA CACTTCACCC CATCACACCC ACACGCCTAC ACACACTCAG

        1101                                                  1150
clone11 GTCTCGCACT CGGGGACCCA TGGGGTGACC CCACGGGCCC AGA.CCAGAG
 clone3 GTCTCGCACT CGGGGACCCA TGGGGTGACC CCACAGGCCC AGACCCAGAG
 clone5 GTCTCGCACT CGGGGACCCA TGGGGTGACC CCACAGGCCC AGACCCAGAG

        1151                                                  1200
clone11 CTGGGTCTTG TGAGCCCTCC CTGTGGACAC CAGCTGGGCC CCACCCTCCA
 clone3 CTGGGTCTTG TGAGCCCTCC CTGTGGACAC CAGCTGGTCC CCACCCTCCA
 clone5 CTGGGTCTTG TGAGCCCTCC CTGTGGACAC CAGCTGGTCC CCACCCTCCA

        1201                                                  1250
clone11 GCGCCCATGG GCTGCTCAGC GGCCCTTTCC CACACTGACC ACACTGACCA
 clone3 GCGCCCATGG GCTGCTCAGT GGCCCTTTCC CACACTGACC ACACTGACCA
 clone5 GCGCCCGTGG GCTGCTCAGC GGTCCTTTCC CACACTGACC ACACTGACCA

        1251                                                  1300
clone11 GGTCAGACAT CCGTTCCTTG CCTCCCCTGG GACACCCACG CCCCTCCCTA
 clone3 GGTCAGACAT CCGTTCCTTG CCTCCCCTGG GGCACCCACG CCCCTCCCTA
 clone5 GGTCAGACAT CCGTTCCTTG CCTCCCCTGG GGCACCCATG CCCCTCCCTA

        1301                                                  1350
clone11 GCAGGCTGAG ATCCCCCCTC AGCCCCTCGT CCTGGCAGCC TCACCCCTCG
 clone3 GCAGGCTGAG ATCCCCCCTC AGCCCCTCGT CCTGGCACCC TCACCCCTCA
 clone5 GCAGGCTGAG ATCCCCCCTC AGCCCCTCGT CCTGGCACCC TCACCCCTCA

        1351                                                  1400
clone11 GGCACAGCAC CCCTCAGGCC CGGTGCTGTC AGCCCTCCCT CCCCGGGGGC
 clone3 GGCACAGGGA CAC...AGCC CGGCGCTGTC TGCCCTCCCT CCCTGGGGGC
 clone5 GGCACAGGGA CAC...AGCC CGGTGCTGTC TGCCCTCCCT CCCTGGGGGC

        1401                                                  1450
clone11 AGGGCCCAGG AACGTGCGCT CTGCTGACCC TCCCAGCTCC AGGCCTGGCC
 clone3 AGGGCCCAGG CTCACATGCT CTGCTGACCC TCCCGGCTCC AGGCCTGGCC
 clone5 AGGGCCCAGG CTCACATGCT CTGCTGACCC TCCCAGCTCC AGGCCTGGCC

        1451                                                  1500
clone11 CCCAGGGCAG AGGAGGCCAG GAACTGAGCC TCTGTCCTGT GGGGAGGTAG
 clone3 CCCAGGGCAG AGGAGGCCAG GAACTGAGCC TCTGTCCTGG GGGGAGGTGG
 clone5 CCCAGGGCAG AGGAGGCCAG GAACTGAGCC TCTGTCCTGG GGGGAGGTGG

        1501                                                  1550
clone11 GGTCAGGGTC CCAGCTCAGG GCACAGCTCA GGATGGGAGC AGGACCCCAC
 clone3 GGTCAGGGCC CCAGCTCAGG GCACAGCTCA GGATGGGAAC AGGACACCAC
 clone5 GGTCAGGGCC CCAGCTCAGG GCACAGCTCA GGATGGGAGC AGGACACCAC
```

Figure 1(c)

```
         1551                                                1600
clone11  AGGCCAGGCC CAGATAGCAG CCAGGGCTGG AGGGGTTGGG GCTGGGGCTG
 clone3  AGGCCAGGCC CAGACAGTGG CCAGGGCTGG AGGGGTGGGG TCTGGGGCTG
 clone5  AGGCCAGGCC CAGACAGTGG CCAGGGCTGG AGGGGTGGGG TCTGGGGCTG

         1601                                                1650
clone11  GGCCCCAGAG ACTGACCTCA GGTGACCCCT GCCTGGCCCA TGGGGAGATC
 clone3  GGGCCCAGAG ACTGACCTCA GGTGATCCCT GCCCAGCCCA TGGGGGGATC
 clone5  GGCCCCAGAG AATGACCTCA GGTGATCCCT GCCCAGCCCA TGGGGGGATC

         1651                                                1700
clone11  ACGCCACCTT CCCCCCACCC AGAGGGAGCC CTGCCC...T ACCCCAGTGA
 clone3  CTGCCACCTT CCCCCCACCC AGAGGGAGCC CTGCCCCGAG GCCCTGATGA
 clone5  CTGCCACCTT CCCCCCACCC AGAGGGAGCC CTGCCCCGAG GCCCTGATGA

         1701                                                1750
clone11  CCCTGCCCAG CCCTCCGTGG GCAGACACAG CACTGACCAC CCCTCCCTGT
 clone3  TGCCACCCAG CCCCCCGTGG GCAGACACAG CACTGACCAC CCCTCCCTGT
 clone5  TGCCACCCAG CCCCCCGTGG GCAGACACAG CACTGACCAC CCCTCCCTGT

         1751                                                1800
clone11  GCAGACTTGC TGCTGGAGGA GGAGATCTGT GCGGACGACC TGGATGGGGA
 clone3  GCAGACCTGC TGCTGGAGGA GGAGATCTGT GCGGACGCCC AGGACGGGGA
 clone5  GCAGACCTGC TGCTGGAGGA GGAGATCTGT GCGGACGCCC AGGACGGGGA

         1801                                                1850
clone11  GCTGGACGGG CTCTGGACCA CCATCTCCAT CTT
 clone3  GCTGGACGGG CTCTGGACGA CCATCACCAT CTT
 clone5  GCTGGACGGG CTCTGGACCA CCATCACCAT CTT
```

Figure 1(d)

Figure 2(a)-(e). Novel nucleotide sequnces 3'prime of the sheep Cgamma genes. Primers are shown in shaded boxes. The 5′ primer is in CH3, and the 3′ primer is in M2. The sequences of clone 11 and clone 1 are set forth in SEQ ID NO: 8 and SEQ ID NO: 9, respectively.

```
         101                                                150
clone11  CCTACGCGTG TGTGGTGATG CACGAGGCTC TACACAACCA CTACACACAG
 clone1  CCTACGCGTG TGTGGTGATG CACGAGGCTC TGCACAACCA CTACACACAG

         151                                                200
clone11  AAGTCGATCT CTAAGCCTCC GGGTAAATGA GCCACATGCC CCCGCACCAG
 clone1  AAGTCGGTCT CTAAGCCTCC GGGTAAATGA GCCACACGCC CCCGCACCAG

         201                                                250
clone11  CAAGCCCTCA CCCAGCCCGC CCTCCCCGGG CTCCAGGTCC AGCCAGGACG
 clone1  CAAGCCCTCA CCCAGCCCGC CCTCCCCGGG CTCCAGGTCC AGCCAGGACG

         251                                                300
clone11  CCCTAGCCCC TCCCTGTGTG CATGCCTCCT GGGCCGCCAT GAATAAAGCA
 clone1  CCCTAGCCCC TCCCTGTGTG CATGCCTCCT GGGCCGCCAT GAATAAAGCA

         301                                                350
clone11  CCCAGGCCGC CCTGGGACCC TGCAACGCTG TGCTTGTTCT TTCCGAGGCA
 clone1  CCCAGGCCGC CCTGGGACCC TGCAGCGCTG TGCTGGTTCT TTCCGAGGCA

         351                                                400
clone11  GAGCCCTGGT GACCGCCAGG CCTGCGGGGG GTGGGCTGAG CCCACTCTGG
 clone1  GAGCCCTGGT GATCGCCAGG CCTGCGGGGG GCGGGCTGAG CCCACTCTGG

         401                                                450
clone11  GCCGCTTGGT TCAGCATCTG TGGGGGCGCT GACCCCTCTC CGGGCCAGAC
 clone1  GCCGCTTGGT TCAGCATCTG TGGGGGCGCT GACCCCTCTC CGGGCCAGAC

         451                                                500
clone11  ACACAGTGAG TGGGTCCGGC AGGGCACCTG GGGGCTGCCC GAGGCCTCGG
 clone1  ACACAGTGAG TGGGTCCGGC AGGGCACCTG GGGGCTGCCC GAGGCCTCGG

         501                                                550
clone11  AGGGGCTTGG CCAGAGGCGC AGCTTCACGG CCCCCTCCAG CCACCACATT
 clone1  AGGGGCTTGG CCAGAGGCGC AGCTCCACGG CCCCCTCCAG CCACCACATT

         551                                                600
clone11  CTGGGCCAGA CTCTGGGCAG GAACGGGGGA AGCCCCCGAC ACCTCAGGGA
 clone1  CTGGGCCAGA CTCTGGGCAG GAACGGGGGA AGCCCCCGAC ACCTCAGGGA

         601                                                650
clone11  TTGAGGCCCA ACGCTTCCCG CCTCTGCTCC AGCCCACGCT GAGGGGCAGG
 clone1  TTGAGGCCCA ACGCTTCCCG CCTCTGCTCC AGCCCACGCT GAGGGGCAGG
```

Figure 2(a)

```
         651                                                 700
clone11  GCCGCGGCCT TGTCCCCAGG CCCCTGTTCC TGGGTGCCCA GAGTCCGTGT
 clone1  GCCGCGGCCT TGTCCCCAGG CCCCTGTTCC TGGGTGCCCA GAGTCCGTGT

         701                                                 750
clone11  GTCCACTCTG GGCCTGCCTG GAGCCAGACT GGCCCAGGGG GAGGCCCTGC
 clone1  GTCCACTCTG GGCCTGCCTG GAGCCAGACT GGCCCAGGGG GAGGCCCTGC

         751                                                 800
clone11  TTCACCCTCA GGCTCCCGAG GTCAGGCATC ATCCTCGTCG GCCAGTAGCT
 clone1  TTCACCCTCA GGCTCCCGAG GTCAGGCATC ATCCTCGTCG GCCAGTAGCT

         801                                                 850
clone11  CTGCCTGGCT CTCTCTGCCC GGGGCCAAGC CTGTGTGCCC ATGGGGAGGT
 clone1  CTGCCTGGCT CTCTCTGCCC GGGGCCAAGC CTGTGTGCCC ATGGGGAGGT

         851                                                 900
clone11  CGTCCCTGTG CCTGAAAAGG GCCCAGGCTG GGAGCCCTGA ACGTCCAGGG
 clone1  CGTCCCTGTG CCTGAAAAGG GCCCAGGCTG GGAGCCCTGA ACGTCCAGGG

         901                                                 950
clone11  CAGGGACCTA GCTGCTCCCT GGGGACACTG AGCCCAGAGC CCCAGACACC
 clone1  CAGGGACCTA GCTGCTCCCT GGGGACACTG AGCCCAGAGC CCCAGACACC

         951                                                1000
clone11  AAGCCCCAGC CCCGCACGCA CACGAGACAG CCCACACCCA GCGTCCTCCA
 clone1  AAGCCCCAGC CCCGCACGCA CACGAGACAG CCCACACCCA GCGTCCTCCA

         1001                                               1050
clone11  CACGCACTCA GGCGTCCACC CGCACACAAG CATGCTTCAC CCCCGTCACA
 clone1  CACCCACTCA GGCGTCCACC CGCACACAAG CATGCTTCAC CCCCGTCACA

         1051                                               1100
clone11  CACCCACATG CCTGCACACA CTCAGGTCTC ACGCTCCGGG ACCCATGGAG
 clone1  CACCCACATG CCTGCACACA CTCAGGTCTC ACGCTCCGGG ACCCATGGAG

         1101                                               1150
clone11  TGATCCCACG GGCCCAGACC CAGAGCTGGG TCTCATGAGC CCTCCCTGTG
 clone1  TGATCCCACG GGCCCAGACC CAGAGCTGGG TCTCATGAGC CCTCCCTGTG

         1151                                               1200
clone11  GACACCAGCT GGTCCCCATT CTCCAGCGCC CTTGGGCTGC TCAGTGGCCC
 clone1  GACACCAGCT GGTCCCCATC CTCCAGCGCC CTTGGGCTGC TCAGTGGCCC

         1201                                               1250
clone11  TTTCCCACAC TGACCACACT GACCAGGTCA GACATCCTTC CTCGCCTCCC
 clone1  TTTCCCACAC TGACCACACT GACCAGGTCA GACATCCTTC CTCGCCTCCC
```

Figure 2(b)

```
         1251                                                1300
clone11  CTGGGGCACC CACGCCCCTC CCTCGCAGGC TGAGACCCCC CCTCAGCCCC
 clone1  CTGGGGCACC CACGCCCCTC CCTTGCAGGC TGAGACCCCC CCTCAGCCCC

         1301                                                1350
clone11  TCGTCCTGGC ACCCTCACCC CTCGGGCACA GGGACACAGC CCGGCACTGT
 clone1  TCGTCCTGGC ACCCTCACCC CTCGGGCACA GGGACACAGC CCGGCACTGT

         1351                                                1400
clone11  CTGCCCTCCC TCTCGGGGAC AGAGCCCAGG CACGTGTGCT CTGCTGAGCC
 clone1  CTGCCCTCCC TCTCGGGGAC AGAGCCCAGG CACGTGTGCT CTGCTGAGCC

         1401                                                1450
clone11  TCCCGGCTCC AGGCCTGGCC CCCAGGGCAG AGGAGGCCAG GAATTGAGCC
 clone1  TCCCGGCTCC AGGCCTGGCC CCCAGGGCAG AGGAGGCCAG GAATTGAGCC

         1451                                                1500
clone11  TCTGTCCTGC GGGGAGGTGG GGTCAGGGCC CCAGCTCAGG GCACAGCTCA
 clone1  TCTGTCCTGC GGGGAGGTGG GGTCAGGGCC CCAGCTCAGG GCACAGCTCA

         1501                                                1550
clone11  GGATGGGAGC AGGACCCCAC AGGCCAGGCC CAGACAGTGG CCAGGGCTGG
 clone1  GGATGGGAGC AGGACCCCAC AGGCCAGGCC CAGACAGTGG CCAGGGCTGG

         1551                                                1600
clone11  GGCTGGGGCT GGGGCCCAGA GACTGACCTC AGGTGACCCC TGCCCGGCCC
 clone1  GGCTGGGGCT GGGGCCCAGA GACTGACCTC AGGTGACCCC TGCCCGGCCC

         1601                                                1650
clone11  ATGGGGGATC ACACCGCCAT CCCCCCCGCC GCAGAGGGAG CCCTGCCCCG
 clone1  ATGGGGGATC ACACCGCCAT CCCCCCCGCC GCAGAGGGAG CCCTGCCCCG

         1651                                                1700
clone11  AAGCCCCGAT GGCCCCGCCC AGCCCCCCGT GGGCAGACAC AGCACTGACC
 clone1  AAGCCCCGAT GGCCCCGCCC AGCCCCCCGT GGGCAGACAC AGCACTGACC

         1701                                                1750
clone11  CCCCTCCCTG TGCAGATCTG CTGCTGGAGG AGGAGAGCTG TGCGGACGCC
 clone1  CCCCTCCCTG TGCAGATCTG CTGCTGGAGG AGGAGAGCTG TGCGGACGCC

         1751                                                1800
clone11  CAGGACGGGG AGCTGGACGG GCTCTGGACG ACTATCTCCA TCTTCATCAC
 clone1  CAGGACGGGG AGCTGGACGG GCTCTGGACG ACTATCTCCA TCTTCATCAC

         1801                                                1850
clone11  GCCCTTCCTG CTCAGCGTCT GCTACAGCGC CACCGTGACC CTCTTCAAGG
 clone1  GCTCTTCCTG CTCAGCGTCT GCTACAGTGC CACCGTGACC CTCTTCAAGG
```

Figure 2(c)

```
        1851                                               1900
clone11 TGGGGGTCCA CCCTGCTGGG CCCTCGGGCC CCCTCTCTGT CCCCAGGGTC
 clone1 TGGGGGCCCA CCCTGCTGGG CCCTCGGGCC CCCTCTCTGT CCCCAGGGTC

        1901                                               1950
clone11 CCCGCAGAGT CCCTCCCTGC CCCTCACTGT CCCTCCCTGT CCCTCTCTGT
 clone1 CCCGCAGAGT CCCTCCCTGC CCCTCACTGT CCCTCCCTGT CCCTCTCTGT

        1951                                               2000
clone11 CCCTCTCTGT CCCTCTCTGT CCCTCTCTGT CCGTTCATTT TCCCTTCACC
 clone1 CCCTCTCTGT CCCTCTCTGT CCCTCTCTGT CCGCTCATTT TCCCTTCACC

        2001                                               2050
clone11 GTAAGCTTGA GACAGATTGG GGTCATTTCA GAGGGCGTCT GAAGAGTCTC
 clone1 GTAAGCTTGA GACAGATTGG GGTCATTTCA GAGGGCGTCT GAAGAGTCTC

        2051                                               2100
clone11 TGTGCCGCAC GCCTCCCTTC ATGTCAGTGG GGAGAATTCA GCAAGGGTGG
 clone1 TGTGCCGCAC GCCTCCCTTC ATGTCAGTGG GGAGAATTCA GCAAGGGTGG

        2101                                               2150
clone11 AGTGCTGGGT GAGAAATGAG GCTTGCGGCG CTCACGAGCA GTGATGGGGC
 clone1 AGTGCTGGGT GAGAAATGAG GCTTGCGGCG CTCACGAGCA GTGATGGGGC

        2151                                               2200
clone11 ACTGCTGCTC CCTGAGACCT GCGCGGACAC CGTTTTCCAT CGCAGGAGAA
 clone1 ACTGCTGCTC CCTGAGACCT GCGCGGACAC CGTTTTCCAT CGCAGGAGAA

        2201                                               2250
clone11 GCGGGCAAGG GAAAACGCCC TCTTGGTCTC TCTTGAGTAA ATGTCGCGTT
 clone1 GCGGGCAAGG GAAAACGCCC TCTTGGTCTC TCTTGAGTAA ATGTCGCGTT

        2251                                               2300
clone11 TTGGTCATCA GTCCCTCCCC CAGTGAGGCT AGAGGAGTTT ACTTCTCCCT
 clone1 TTGGTCATCA GTCCCTCCCC CAGTGAGGCT AGAGGAGTTT ACTTCTCCCT

        2301                                               2350
clone11 CTCGATGGTC AGGTCAGGAC TGTCATAGAC TCCGGATCAC CTTCCTGTAA
 clone1 CTCGATGGTC AGGTCAGGAC TGTCATAGAC TCCGGATCAC CTTCCTGTAA

        2351                                               2400
clone11 ATGCTTGCTT TTTGTGTGCA GAGAGCCTGT TTTAGCTCGG GGGTCCTCAG
 clone1 ATGCTTGCTT TTTGTGTGC. .AGAGCCTGT TTTAGCTCGG GGGTCCTCAG

        2401                                               2450
clone11 CTCACTGAGC TCGCGGGGCA GGGGTGGGCT CGGGCTGGCG CCGCCTGTTC
 clone1 CTCACTGAGC TCGCGGGGCA GGGGTGGGCT CGGGCTGGCG CCGCCTGTTC
```

Figure 2(d)

```
        2451                                               2500
clone11 GGGAGCGCAT CTCCAGCATG CTGTCGCACA GCTTCGTTGC TAACAAGACC
 clone1 GGGAGCGGCA TCTCCAGCTG CTGTCGCACA GCTTCGTTGC TAACAAGACC

        2501                                               2550
clone11 GCTTAGTCTC GTGGTTAGAC CAACCTGCTT TCTCGAGTAA TTGTTAATTT
 clone1 GCTTAGTCTC GTGGTTAGAC CAACCTGCTT TCTCGAGTAA TTGTTAATTT

        2551                                               2600
clone11 ACAGGAGTTT CCTGTATTTT TCAACTTATA ATCCCCTAGT CAGATAACTC
 clone1 ACAGGAGTTT CCTGTATTTT TCAACTTATA ATCCCCTAGT CAGATAACTC

        2601                                               2650
clone11 TTTAATCACC TATTCTGCCC CTTCATTTTC TCCCTATCGA TCTCAGCAAC
 clone1 TTTAATCACC TATTCTGCCC CTTCATTTTC TCCCTATCGA TCTCAGCAAC

        2651                                               2700
clone11 CCATCACTGC CCTCACTGTC CTTAAACTGT CCCTTAACTG ACCAGACTGT
 clone1 CCATCACTGC CCTCACTGTC CTTAAACTGT CCCTTAACTG ACCAGACTGT

        2701                                               2750
clone11 CCCTCAGTGT CCCCTCAGAG TCACCTCCCT ATCACCTCAC TGTCCCTCTC
 clone1 CCCTCAGTGT CCCCTCAGAG TCACCTCCCT ATCACCTCAC TGTCCCTCTC

        2751                                               2800
clone11 TGCCCCTCTC TGCCCCTCTC TGTCCCTCCC TGCCCCTCCC CGTCCCCTCT
 clone1 TGCCCCTCTC TGCCCCTCTC TGTCCCTCTC TGTCCCTCCC CGTCCCCTCT

        2801                                               2850
clone11 CTGTCCCTCT CTGCCCCTCA CTGCTCCTCT CTGCACCTCA CTGCTCCTCA
 clone1 CTGTCCCTCT CTGCCCCTCA CTGCTCCTCT CTGCACCTCA CTGCTCCTCA

        2851                                               2900
clone11 CTGCCCTGGG GGAGGCCCGC ATCGAGGTGT CTCTGCTCAC CCCGTCCCCC
 clone1 CTGCCCTGGG GGAGGCCCGC ATCGAGGTGT CTCTGCTCAC CCCGTCCCCC

        2901                                               2950
clone11 ACCCCGTACC CCCCGCCAGG TGAAGTGGAT CTTCTCCTCG GT
 clone1 ACCCCGTCCC CCCCGCCAGG TGAAGTGGAT CTTCTCCTCG GT
```

Figure 2(e)

Figure 3(a)-(b). Novel 3'prime flanking sequence (SEQ ID NO: 10) of rabbit Cgamma gene.

CTCCCCCCCACGCCGCAGC
TGTGCACCCCGCACACAAATAAAGCACCCAGCTCTGCCCTGAGAGGCTGTCCTGATTCCT
TCCAAGGCAGAGGCTTCCACTCGGGCCGGACAGGGTTGGGCGGGCGCCGTGGGCTCTGCT
GTGGCCAGCAGCCAGAACGGTCAACAGTGGGACAGGGGCAGACCCACAGCACAGGGGCCT
GCCAAGAACTGGGCTCAGCCGGAGTGCTGTGGCAGGTCCCCCCTTGCAGCTAGCACGTGT
GTGCTGGGCAGGCAGAGGCCCCCAGGGGAGGAGCACACAGCTACCACCTCTGCAAGAGCC
TGGCCTGGCGCCCAGGTCCCAGTCCACAGGGTGTGTAGTACACAGAGCCTCATCTTACCA
CAGATGTAGGGACAGACCCACCACGCCCCTGCACCCCACCCAGCCTCGCCCCTTGTGGGA
CCAGGGCTACCACTCCACTCCCCCGCCCAGAGCAGCAGAAGCAGGTGGCATCCTCAGCAG
AGGGACAGTCTCACCCCTCCACGGCACTGAGCCCTGACCCATCAAACAAGCCCCTCCTGC
TGCACAGCACCTGTGTGCACATCACACACACACACACACACACACTGAGGCCTGACCCCA
TCAAACAAGCCCCTCCTGCTGCACAGCACCTGTGTGCACATCACACACACACACACACAC
ACACTGAGGCCTGACCCCATCCTGCCCTCCTGCTGCATGGCACCTGTGTGCACATCACAC
ACACATGCACACACACACTCACACACACTGAGCCCTGACCCCATCCTGCCCTCCTGCTGC
ATGGCACCTGTGTGCACATCACACACACACACACACACACACACACACTGAGCCCTGACC
CCATCCTGCCCTCCTGCTGCATGGCACCTGTGCACACATCACAAACACGCCTGCCTCATA
CACTGGCACTCAGAAGGGGCCCCTGTACACGCATACACATGCACACACCTTGACACATGG
GCCCCCTACACACGCATCACACACACTCATGCACACTCCTCACACATGGCCCTCCTGCAC
ATACATTGCACACACATGTGCACAGACCTCACAATGGGCCCCTGCACACACATTGTACAC
ACGCATGTGCACACACTTCACACATGGGCCCCCTGCACATGCATTGCACACACAGACACA
CACATGTGCATTCCTCACACATTGGGGCCTTGCAAGGGATGCCCTGCACACACATTGCAC
ATGCTCACATGTGCACACACCCCACACTGGAGCCTTGCATAGGGCCCCCTGTACACACAC
CATGCATACACACACACCTCACACAAGGGGCCCCCTACATACGCAAAACACACACACACA
CATGCACACACCTCATACACGGGCCCCCTACACACATCACACACACACACACACACACGT
ATGCATGCCTCACACACAGACCTTGCAAGGGGCCCCCTGCACATGCATCAAACACATATG
CACATGTTTCACACACACGGTCCCCTACACACACTGCACACGCACACATGTGTACATGCT
TCACACACTGGGGCCTTGCATGGGGTCCCCTGCATAGCATAGCACCCAGAGCCACGCCAG
GTGCCTGGGCACATGGACACTGGTGCACACACAGCACCCAAGCCCAGCTCTCCCATCCAA
GGGGCACCAGCACCCCCCACTCACGAGCACCCTGAATTCCTGCTCCCCACAAGCGAACGT
GCACCCTACCTCTCCAGACGTCCCTTTCCTGTGGCCACTCCCATAGGTATTGGCGAGACC
CTCCCTTGACCCTTGGGCCTGGTCACCCAGGGGACAGGAGAGGGCCAAGTTGGGCCACAG
TACCACTGCCCAGCAGGGGTGAGGCAAGCAGAGGGTGGGTCTGTGAGGCGTCTGGCCAGC
CGTGCTGGGGCCCAGGTGGGGAGCAGCTGGGTGGCTGAGGTGGCTTCCTTGCAGGTGGTT
GGGGGGAGCTGGCCCCACAAGTGCCACTGCCCAGCACTGTCCAGTGCTTCCCCCTGAACC
TCCCGGCCACCCATCCCCAGCTGCAGCCGCAGAGGGAGTGCCCCTCGGCCTCCTCGGCAA
GACGCACGCTGACTGCCCCTCCCCATCCAGAGCTGCAGCTGGACGAGAGCTGTGCCGAGG
CCCAGGACGGGGAGCTGGACGGGCTGTGGACCACCATCACCATCTTCATCTCCCTCTTCC
TGCTCAGCGTGTGCTACAGCGCCACAGTCACCCTCTTCAAGGTGGGTGCTGCACCCGGCA
CGGGTGGGCTGGGGGCCAGGGGCGGGGGCCGGGGGCCAGGCCCTCCTCACCCCGCGCCGC
CGCTGCTGCAGGTGAAGTGGATCTTCTCGTCCGTGGTGGAGCTGAAACACACCATCGCTC
CCGACTACAGGAACATGATCGGGCAGGGGGCCTAGGCCCTTCGTTCTCACAGCCTGCCTC
CCTGGCCAGCAGGAGCCCCCGCCTCCGCCTCGGACCCCATGGCTCTCTGCTCTGGCCGCT
CCGGACCCCTCCGCCTCGGGAGAAGCGCGCAGCTGATGCCTGCCGGCCCCTCCACGCAGC
AGTGCGGACAGCACGCATCTGTCGTCCACCCGGCAGGACCCCACCCAGGGCCAGCCCTGA
CCGCCAGCCTCCTGGACTCAGGGCTCCTCTGAGAAAAGGCCCACTTGTTGGTCCCCTCAG
CCCACACCCAGGCAGCCTCCGGTGGGTGCTTCCCTGGACCCCAGCCTGAGGCCTATGCTT
GTTCTCCTGTGGCTCTTACTCAGAGGCCCGTGCTGGACTCCCACCCACAGGGACAGTGCC

Figure 3(a)

CTGCTCCAACCCTCACTGCACTGGGGGTCATGGGGCCACCTTCTGTGCAGGGGTCCTGGC
TCCAAGGAGAACACTCGAAGGGCCTGCTTGGCCACCTGGCACCACGGGAGCCCCGCTGGG
TAGCTTGGCAGGGACCCCTGAGTAGAGGTGGGTGCACCCAGCCAGAAAGCCTGCTGGATG
GACAGGAGCCTGGCGTCCGGGCCCCAGGCAGGCAGACACGGCTTCATGGACAGGAGAGGC
CAAGGAACATCAGCAAAGAGAGACAGCTGGGCCGGGCGTTCCAGCCAGACCCATCCTGCA
GCCCAGCATCGGCCCCGTGTACTACAGCAGGGACAGCCAGT

Figure 3(b)

Figure 4. Novel nucleotide sequence (SEQ ID NO:11) 3'prime of the rabbit Ckappa 1 gene.

GCGAGACGCCTGCCAGGGCACCGCCAGTGACCCTGAG
GCCCAGCCTCGCCGCTCCCTCCCCTCAGTGGACCCATTCCCACCACAGTCCTCCAGCCCC
TCCCCTCCCGGCCCTCACCCCCTCCTTGGCTTTAACCTTGCGAATGTTGGTGAGATGGAT
GAATAAAGTGAATCTTTGCACTTGTGACTTCTCTCTGCTTCTTCATTTAATGGTTATTAC
TCATGGTTTCCCAGTTGCCCTAAAGTCACCGCCATTTCATCCTCCATCCCACCCTGCCCT
GCTGTCCTCCGGGAGACACCACTCCCTGAAACCCACAGGCCCCTGTCTTCACACCGCCGA
CCCCGACCACACGTGAGGGGCTTGCTTCGTGTCTCACTCCCCTCATCGAGCCCCAGAGTC
CTCCTTTAGTGTTCTTACAGTCACATACAGTTATACAGTTTGAGTCAATCCAACCTGCCC
TGCCAATTTCCCAAAACAAAGATTTTCAGAATAAAACAGCTATGAAGAAAGTCATTTATG
GAAGCATGATATACAACAACAAAACAATGCAAACAACCTAACTGAATAAGCAGAGGGAAA
TGTTCAGACACACTATGGGGCTTGGGCTTCATGGAGTATTACACCTTCATTACATTTTTA
AACTTGTATTAAGGAGCTCCTATATTACAAGGATTATACTAGAGCACTTTCCATGACCTA
ATTAATTCTCATTACACTGTGAGGTTAAAAGCATTAGTTAAAATATTGGGCAGGCTCCCT
ATAGCCAACAGTTGTTCATATTCCATAACCCAACCATCATTTAGGTGACTCAGGGTCCTT
GTCCACCAAGAACTTTGGCAAGAATGTTCAGAGCAACTTCCTTTATAAAAGTCAAAAATT
GGAAGTAACTCAAATGTCTACCAACAGTAGAATGGGCTGTTAATTGGCATATGTTTACAT
ATTAGAATGCTGTTTAATAAAGAGAATTAACAAACTACAACTATCCCTAATAACATAGGT
GACTCATAAACATGATGTTAAGCACAAGAACCCAAACACAAAAGACACACTGTGTATGTT
TTCATCCATAGGAAGTTCAAAACTAGTTAAAAATTGAATTAGAAATTGAGATGAAGTTTA
CTCTTGGCTGGGGGTGTGGAGTGAGGCGGTGCCTGGTGGGGGACAGAAAGTGGCTGCTGG
GGTCTTGGTGATGTTCTAGTCCTCACTGTGGTGTGTGCTACTCTGAAAATGTATTGAGTA
CACAATTAGGTTTTGTGCTTTCATTATACTCCAAAGTAAGTTCTCATAAACATTGCCTTA
CACGGGGTCTACAGATAAGAGAGACTAAGAGGAATGAGTAACAGATCAAGGCCACACAGC
TGGTAGGCATGGGCCTGGGATCAAACCCTGTCTGCCCAATTCTGCTCTCTTGAGCCCTAC
ACTATTCTTTCCAGCACTGGAATGCCATGCAGAACAGGGAGTAGGACATGCTACCTCCCT
AGGGTCTCCTCCTTTACCCACCTAACCAGGAGCACCCATACATAGAAACAGGATGGAAAA
GACCATCAGCAATGGAACAAGGGAGAGATTAACCTTGTTCAGTATTGTGATCCCATGTAG
GAAAGATTGTGGGAGGAGGGCTGCACACAGAGCACCGTCCCCCTTCTATGTGCCCACCGC
TCTGTGCCCCCTTATCTGCTCACCCGCCCAGCGTGCATTCACTCAGCACCCTTTTCGCCT
GCCCTCTGAAAGAGGTGCAGAAGTAACTAAACCAGCTTCCCTCCTTCAGTGACTTGGAAT
CCAGTTTTCCTCCTCTATTTCCCCCTCCTTTTCAGTGCAGGAGCCTGGAGAAATGTGATT
TGTGTTATTATAAATTTCCCACATCATTTTGTGTAAGGGAAAATATACTCAACAGTCATA
ACTGGTAAAACTGCTGTGAAAACTAAGAGAAGTAATTCATGCGAAGGTTGAGCACCAGCC
TTGTATATACTAAGAGATCCAGAAGTGTTAGTCACCGTTAGAAATAAGAAGGAGTAGCTC
AATTTGACTAGTTCCTGGTTCACTCCTTGAACATGTTCTTCAGTTATCATCTTTCAGTCC
CAAATGATTGAACTTGGAATTAACTCACATGGATTCTAGACCTGTGCCGAGAATGGCTGC
CACTCGTGCTCTAGAGCTCTGGGGATGAGGCTGTCCCTACTGTGGTGTGCTACAGGTCTA
ACAACACACCAGGTTTTGAAGACTTAGCACTATGAATATATATATATATTATATTCCAAT
AAATTTAACATACTTTCTACTTTCATTGCATGTTGAGATAGTAATCTACTTTGGATATAT
TTGGTTAAACCAAACTATTCTCAAGACAAATTTCATAGGTTTATGGTTTTTTTACAATTT
AATCAAAATATAAACATAGTCCAAACAATTAATCCATTTAAAGTGGAGAATGGCCCAAGT
GTTTGGGCCCCTGCTACCCATTTTTAAGACCAGATGTTGCTCTTGGCTTCTGGCTTTTGC
TTGGCTCAGCCCTGGCCATTGCAGCCATCTGAGGAGTAAACAGTGGATGGAAGACATCTC
CCCCCACCCTGCCCATAAAGCTCGGGATCC

Figure 5. Novel nucleotide sequences (SEQ ID NO:12 and SEQ ID NO: 13) 5'prime of the rabbit *Cgamma* gene. The sequences between SEQ ID NO: 12 and SEQ ID NO: 13 (a gap of about 1000 nt) remain to be determined.

CTTCAGCGTGAACCACGCCCTCCCGCTGAGTCTCACGCACCCCCG
GTGCAGGCAGTCCGGCCTCACCTGGAAGGTGCACTGACTGAAGACACTGCAAGGGGTGAG
AGCATTTCTCAGGAAAGAGCCCTGAGTTTAGAAGGCCAGAGAGCAGAGGGCTGAGGGCTG
CCTTGCGCTGCAACCCATGGAAACACAGGCTTAGCAGATGTTCAAGCTCCGGGAGTCCAC
ACTGGGTGAGGGCAGGCGTCCAGCCTGACATGGCCCCCACAGACTCGCCCACAGGTGACG
CCAGATGAGGACGGTCAAGGATCGGGGGATCCTACATGCCCAGGGGCACCAAGACAGCCA
GGAGAGCACCAGAGGCCACAAGAGAGGCCTGGGACAGTCTCCCTGCTGACATCCAGAGCC
CAGGCCCCACTTGGCAGAGCTGGCTGAGAACACGTCTCTGCGGTGGAAGCTGCCCCGTCC
TGGGTGTTGCTCGGCGGGCTAAGCCGACTGACGCGGGGCGGGCCAGGCCATCGGCCCCAC
GGCCTGCAGCTTCCTCCCCAGCCCAGGCCACGTGGGCTCCTGGCTGAACTGGCCGCTCGC
TGAGCTCTCACCCCCCCACCCAGCAGCAGGCCGGGCGGTGCTGCCATGAGCTCCATTCCC
ACCACACAAGCGACAGCCCGGGCAGCGCCCCAGGCCCACGGGGCGTTTGCTGTGCGGCTC
GCACTCGCTGCTCAGGGCCAGCGCAGGGTGCAGCAGGGACTCACCAACCCGCCCCGACTC
GGCTGGCACGTTTACTGGAGGCCTCTGAGCCTGACCGTGGCAGTGGGGCCCGAGCAGGCT
CCAGGCTGCCCCCTGCACCCTGGGCTTGCCGCTCCGGGACCCCTGGTGGGCACCTTCCCA
GATGTGCTCCCACCGTGCCTCCTTGGGGCTCTGGGCTCATAGCGGTCACTCTCCGCCTTC
TCTCCTCCCAGCCCTTTCCTGCCTCCCTATGGCCCCATCTAGCTCTGCCCTNTCTAGAGC
CTCTACCTGGAAGGAATCTGCTGTTGGACCAAGACACCACCCGCAGCACAGGTGGGCGCC
TTGCACTGTGCTAGGCCCTCCCCGCACAGAAAAGGGCCCTAGGCTCTGGAGGCTGCTGCT
GNCTCTGGGGCTGGCATCGGGCGCACCCTGCACCCTGCACCCTGAGGAAACTCAGGCCTG
CCCGCTCCAGGCCTGTCCCT

Gap of about 1000 nt

GAAGCTTTACTTGTTGGGGGCGG
GCAGGTCTAAGGGACCTGCCAGGTGTGGGGGCTGGGCTTGACTCAGCAGGAGCCTTCTAG
AAGGAAAGCTCTGGAGAAGGTGGGGGCAGAGGGCGGGAAAGGCCTGTGAGGAGGCGGGTG
GTGGGCAGGGCCACTGGGAAGGGAGGGCTGGGGGTGACACTCAGGTTGGCACTGGGGAGG
ACCTGAGGAGGCAGGTGCCAGGCACAGAGCTGAACCTGGGCAGGGCAGGGGCAGGTAACA
AGAAGGATTCTCCTTGGAGCCTGGTCCAGGGTGGTCCAGGGCGGTCCAGGGCCTGGGGTT
TGCAAGCTGGGCTGTGACAGGGCCTCTCTCCCCAGGGGCAAGCAGCAAAGCCTGGGCACA
GAGCCCAAAGCCCCCACACAGAGAAGCTCCCCAGGGCAGGGCCTGCAGGGCTTGGGGGAC
CTTCTTGGAGCAGGCAGAGGACAGAGGCATGAGATCAGCCTCCCAGAGGCTGGAATGATA
GGTCCAGCAGGAGGGGCCCACATGGGCTCTGGTTAGCAGGAGAAAACAGCCCCCAGGTCC
CCATGGCCACCACGCACCGACTGCTGGTGAAGCTTTGGGTGGCAGACGAGAGCCACATGG
CAGCTGCTCCTGTCACTCTCTGGCAGTGCATCGAGGGCGTCGAC

Figure 6. Comparison of human, mouse, rabbit, sheep, cow and camel sequences for the the M1 and M2 regions 3′ of the Cgamma gene.

M1

	1				46	SEQ
camel	EPLLEEESCA	EAQSGELDGL	WTTISIFITL	FLLSVCYSAT	VTLFK.	14
human-Ig3	.ELQLEESCA	EAQDGELDGL	WTTITIFITL	FLLSVCYSAT	VTFFK.	15
human-Ig3/2	.ELQLEESCA	EAQDGELDGL	WTTITILITL	FLLSVCYSAT	VTFFK.	16
human-Ig1	.ELQLEESCA	EAQDGELDGL	WTTITIFITL	FLLSVCYSAT	VTFFK.	17
mouse-Ig1	.GLQLDETCA	EAQDGELDGL	WTTITIFISL	FLLSVCYSAA	VTLFK.	18
mouse-Ig2a	.GLDLDDVCA	EAQDGELDGL	WTTITIFISL	FLLSVCYSAS	VTLFK.	19
mouse-mRNA	PGLQLDETCA	EAQDGELDGL	WTTITIFISL	FLLSVCYSAA	VTLFK.	20
mouse-Ig3	.ELELNETCA	EAQDGELDGL	WTTITIFISL	FLLSVCYSAS	VTLFK.	21
mouse-Ig3/2	.ELELNGTCA	EAQDGELDGL	WTTITIFISL	FLLSVCYSAS	VTLFK.	22
sheep-clone11	.LLLEEESCA	DAQDGELDGL	WTTISIFITP	FLLSVCYSAT	VTLFK.	23
sheep-clone1	.LLLEEESCA	DAQDGELDGL	WTTISIFITL	FLLSVCYSAT	VTLFK.	24
cow-clone11	.LLLEEEICA	DDLDGELDGL	....	....	......	25
cow-clone3/5	.LLLEEEICA	DAQDGELDGL	....	....	......	26
rabbit	..LQLDESCA	EAQDGELDGL	WTTITIFISL	FLLSVCYSAT	VTLFK.	27

M2

	1		27	SEQ
camel	VKWIFSSVVE	LKRTIVPDYR	NMIGQGS	28
human-Ig3	VKWIFSSVVD	LKQTIIPDYR	NMIGQGA	29
human-Ig3/2	VKWIFSSVVD	LKQTIIPDYR	NMIGQGA	30
human-Ig1	VKWIFSSVVD	LKQTIIPDYR	NMIGQGA	31
mouse-Ig1	VKWIFSSVVE	LKQTLVPEYK	NMIGQAP	32
mouse-Ig2a	VKWIFSSVVE	LKQTISPDYR	NMIGQGA	33
mouse-mRNA	VKWIFSSVVE	LKQTLVPEYK	NMIGQAP	34
mouse-Ig3	VKWIFSSVVQ	VKQTAIPDYR	NMIGQGA	35
mouse-Ig3/2	VKWIFSSVVQ	VKQTAIPDYR	NMIGQGA	36
rabbit	VKWIFSSVVE	LKHTIAPDYR	NMMGQGA	37
sheep-clone1/11	VKWIFSSV..	....		38

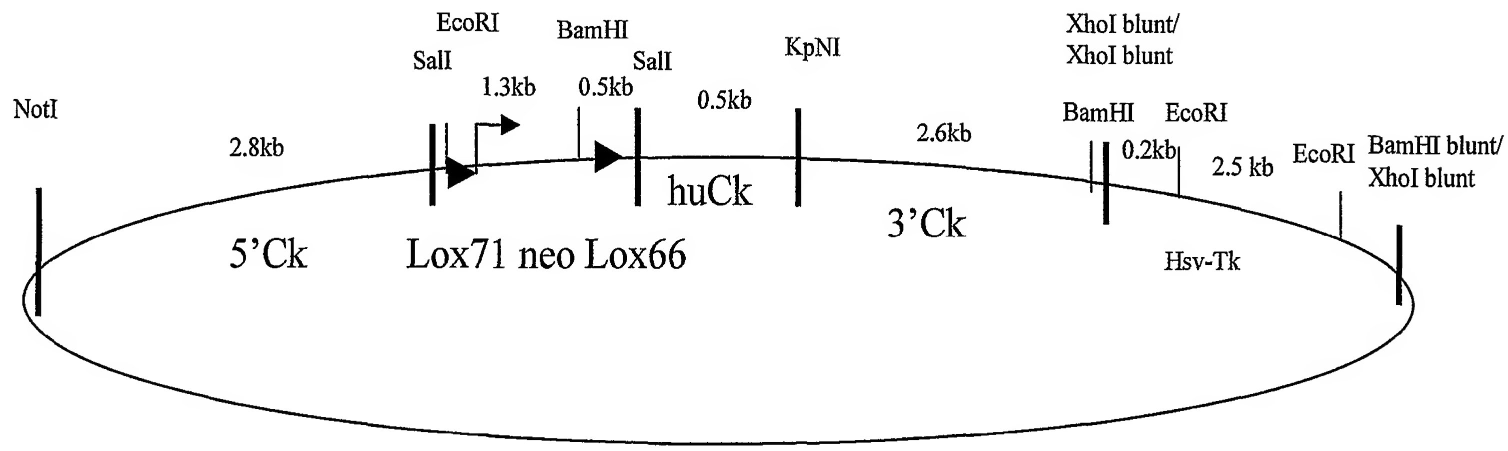

Figure 7a: DNA construct for the replacement of rabbit Cκ with human Cκ. A 0.5 kb fragment containing a DNA sequence encoding human Ck is flanked by sequences from the rabbit Cκ1 gene. The upstream sequence (5'Cκ) is 2.8 kb, the downstream sequence (3'Cκ) is 2.6 kb. The vector also contains a lox-neo cassette for positive selection and a Hsv-Tk casette for negative selection.

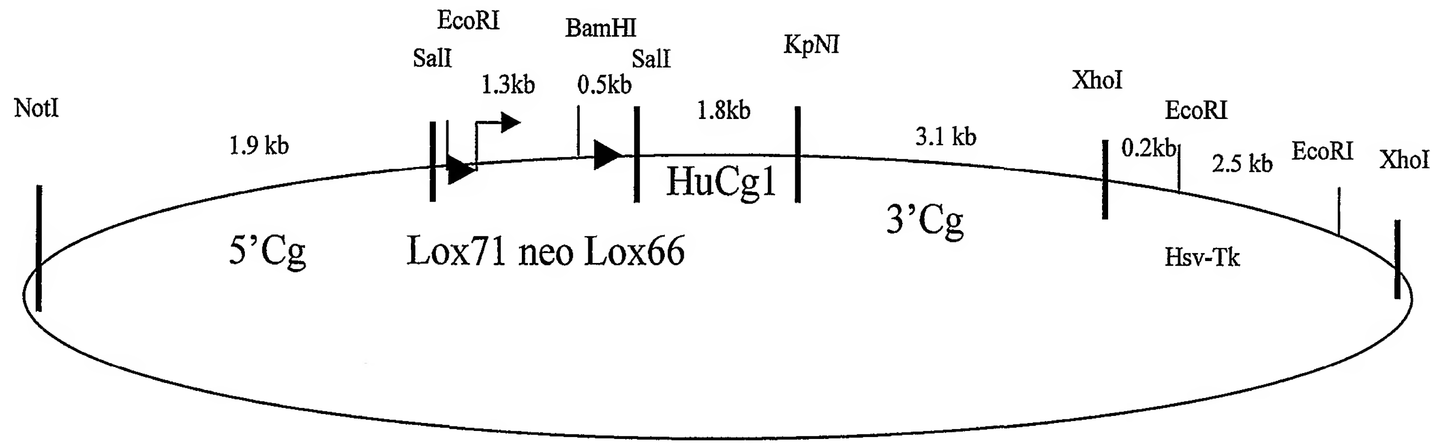

Figure 7b: DNA construct for the replacement of rabbit Cγ with human Cγ1. A 1.8 kb fragment containing a DNA sequence encoding human Cγ1 is flanked by sequences from the rabbit Cγ gene. The upstream sequence (5'Cγ) is 1.9 kb, the downstream sequence (3'Cγ) is 3.1 kb. The vector also contains a lox-neo casette for positive selection and a Hsv-Tk cassette for negative selection. The figure is not up to scale.

Figure 8. DNA fragment (SEQ ID NO: 51) containing a human immunoglobulin heavy chain Cγ1 gene segment flanked by 50 nucleotides derived from the rabbit heavy chain immunoglobulin gene. Flanking sequences derived from rabbit immunoglobulin DNA sequences are underlined.

```
                      tgacctacct accctgccaa ggtcaggggt cctccaaggc
aagggatcac atggcaccac ctctcttgca gcctccacca agggcccatc ggtcttcccc
ctggcaccct cctccaagag cacctctggg ggcacagcgg ccctgggctg cctggtcaag
gactacttcc ccgaaccggt gacggtgtcg tggaactcag gcgccctgac cagcggcgtg
cacaccttcc cggctgtcct acagtcctca ggactctact ccctcagcag cgtggtgacc
gtgccctcca gcagcttggg cacccagacc tacatctgca acgtgaatca caagcccagc
aacaccaagg tggacaagaa agttggtgag aggccagcac agggagggag ggtgtctgct
ggaagccagg ctcagcgctc ctgcctggac gcatcccggc tatgcagccc cagtccaggg
cagcaaggca ggccccgtct gcctcttcac ccggaggcct ctgcccgccc cactcatgct
cagggagagg gtcttctggc tttttcccca ggctctgggc aggcacaggc taggtgcccc
taacccaggc cctgcacaca aaggggcagg tgctgggctc agacctgcca agagccatat
ccgggaggac cctgcccctg acctaagccc accccaaagg ccaaactctc cactccctca
gctcggacac cttctctcct cccagattcc agtaactccc aatcttctct ctgcagagcc
caaatcttgt gacaaaactc acacatgccc accgtgccca ggtaagccag cccaggcctc
gccctccagc tcaaggcggg acaggtgccc tagagtagcc tgcatccagg gacaggcccc
agccgggtgc tgacacgtcc acctccatct cttcctcagc acctgaactc ctggggggac
cgtcagtctt cctcttcccc ccaaaaccca aggacaccct catgatctcc cggacccctg
aggtcacatg cgtggtggtg gacgtgagcc acgaagaccc tgaggtcaag ttcaactggt
acgtggacgg cgtggaggtg cataatgcca agacaaagcc gcgggaggag cagtacaaca
gcacgtaccg tgtggtcagc gtcctcaccg tcctgcacca ggactggctg aatggcaagg
agtacaagtg caaggtctcc aacaaagccc tcccagcccc catcgagaaa accatctcca
aagccaaagg tgggacccgt ggggtgcgag ggccacatgg acagaggccg gctcggccca
ccctctgccc tgagagtgac cgctgtacca acctctgtcc ctacagggca gccccgagaa
ccacaggtgt acaccctgcc cccatcccgg gatgagctga ccaagaacca ggtcagcctg
acctgcctgg tcaaaggctt ctatcccagc gacatcgccg tggagtggga gagcaatggg
cagccggaga acaactacaa gaccacgcct cccgtgctgg actccgacgg ctccttcttc
ctctacagca agctcaccgt ggacaagagc aggtggcagc aggggaacgt cttctcatgc
tccgtgatgc atgaggctct gcacaaccac tacacgcaga agagcctctc cctgtctccg
ggtaaatgag cgctgtgccg gcgagctgcc cctctccctc ccccccacgc cgcagctgt.
```

Figure 9. The DNA fragment (SEQ ID NO: 52) containing a VH gene segment with more than 80% sequence identity with rabbit VH elements and encoding a human VH element polypeptide sequence. Flanking sequences derived from rabbit immunoglobulin DNA sequences are underlined.

```
           tgagtgacag tgtcctgacc atgtcgtctg tgtttgcagg tgtccagtgt
gaggtgcagc tgttggagtc cgggggaggt ctcgtccagc caggggggac cctgagactc
acctgcgcag tctctggatt caccttcagt agctatgcaa tgagctgggt ccgccaggct
ccagggaagg ggctggaatg ggtcggagcc attagtggta gtggtagcac atactacgcg
gacagcgtga aaggccgatt caccatctcc agagacaact ccaagaacac gctgtatctg
caaatgaaca gtctgagagc cgaggacacg gccgcctatt actgtgcgaa agacacagtg
aggggccctc aggctgagcc cagacacaaa cctccctgca
```

Figure 10. DNA fragment (SEQ ID NO: 53) containing a human immunoglobulin light chain Cκ gene segment flanked by 50 nucleotides derived from the rabbit light chain immunoglobulin Kappal gene. Flanking sequences derived from rabbit immunoglobulin DNA sequences are underlined.

```
                                            .catccacat ggcacccagg
ggagatgtcc actggtacct aagcctcgcc atcctgtttg cttctttcct caggaactgt
ggctgcacca tctgtcttca tcttcccgcc atctgatgag cagttgaaat ctggaactgc
ctctgttgtg tgcctgctga ataacttcta tcccagagag gccaaagtac agtggaaggt
ggataacgcc ctccaatcgg gtaactccca ggagagtgtc acagagcagg acagcaagga
cagcacctac agcctcagca gcaccctgac gctgagcaaa gcagactacg agaaacacaa
agtctacgcc tgcgaagtca cccatcaggg cctgagctcg cccgtcacaa agagcttcaa
caggggagag tgttagagcg agacgcctgc cagggcaccg ccagcgaccc tgaggcccag
cctcgc.
```

Figure 11. DNA fragment (SEQ ID NO: 54) containing a Vκ gene segment with more than 80% sequence identity with rabbit Vκ elements and encoding a human Vκ element polypeptide sequence. Flanking sequences derived from rabbit immunoglobulin DNA sequences are underlined.

```
                                            ..ggcaggct gctcccaccc
catgcaggag gcagtaccag gcaggaccca gcatggacat gagggtccct gctcagctcc
tgggactcct gctgctctgg ctcccaggta aggagggaaa caacaaaaat tttattcagc
cagtgtagcc actaatgcct ggcacttcag gaaattcttc ttagaacatt actaatcatg
tggatatgtg tttttatgtt cctaatatca gataccagat gttacatcca gatgacccag
tctccatcct ctctgtctgc atctgtggga gacagagtca ccatcacttg ccgagccagt
cagggcatta gcaattactt agcctggtat cagcagaaac cagggaaggt tcccaagctc
ctgatttatg ctgcatccac tttgcaatct ggggtcccat cgcggttcag tggcagtgga
tctgggacag atttcactct taccatcagc agcctgcagc ctgaagatgt tgccacctat
tactgtcaaa agtacaacag tgcccctcca cttttcggcg gagggaccaa ggtggagatc
aaacgtaagt gcactttcct aatgttcctc accgtttctg cctgatttgt ttgctttttc
catttttcgctat..
```

Figure 12. DNA fragment (SEQ ID NO: 57) containing a gene encoding human immunoglobulin light chain constant region Clambda2 flanked by 50 nucleotides derived from the chicken light chain gene. The DNA sequence of chicken origin is underlined.

catacacag ccatacatac gcgtgtggcc gctctgcctc tctcttgcag gtcagcccaa
ggctgccccc tccgtcactc tgttcccgcc ctcctctgag gagcttcaag ccaacaaggc
cacactggtg tgtctcataa gtgacttcta cccgggagcc gtgacagtgg cttggaaagc
agatagcagc cccgtcaagg cgggagtgga gaccaccaca ccctccaaac aaagcaacaa
caagtacgcg gccagcagct atctgagcct gacgcctgag cagtggaagt cccacagaag
ctacagctgc caggtcacgc atgaagggag caccgtggag aagacagtgg cccctacaga
atgttcatag tagtcccact ggggatgcaa tgtgaggaca gtggttcctc accctccctg

Figure 13. Modification of the chicken light chain locus using the ET system.
A chicken genomic BAC clone with the full length light chain locus was modified by homologous recombination. In a first step Cλ was deleted by insertion of a selection cassette which was in a second homologous recombination step exchanged against the human Cλ gene. The homology stretch was 50bp.

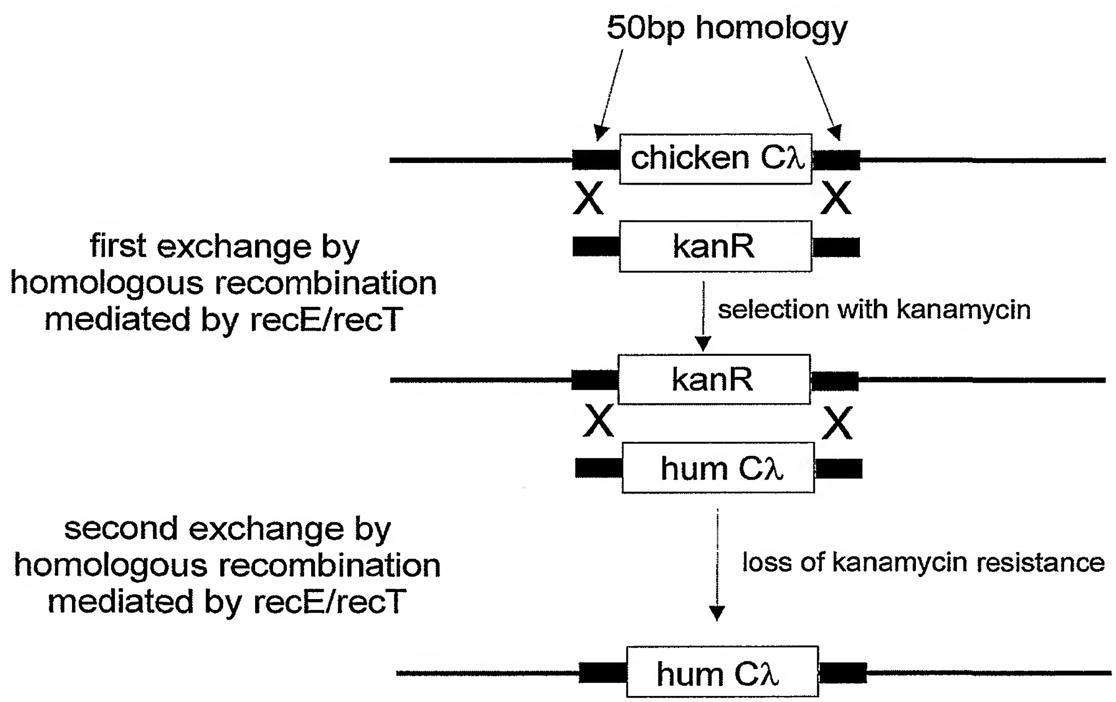

Figure 14. DNA fragment (SEQ ID NO: 58) containing a VJ gene segment with 80% sequence identity with chicken V gene segments and encoding a human VJ immunoglobulin polypeptide. Flanking sequences derived form chicken immunolgobulin DNA sequences are underlined.

```
.ttgccgttt tctcccctct ctcctctccc tctccaggtt ccctggtgca gtcagtgctg actcagccgc
cctcggtgtc agcagccccg ggacaagaag tcacgatctc ctgctccggg tctagtagca acattggcga
taatttcgtc tcttggtacC agcagctgcc tggcactgcc cctaagcttc tgatctatga taacaacAag
agaccctcgg gcatccctga ccgattctcc ggttccaaat ccggcacctc agccacatta ggcatcactg
ggctccaaac cggcgacgag gctgactatt actgtgggac ttgggacagc agcctttctg ttggtatgtt
tgggggcggg acacgcgtga ccgtcctagg tgagtcgctg acctcgtctc ggtctttctt cccccat...
```

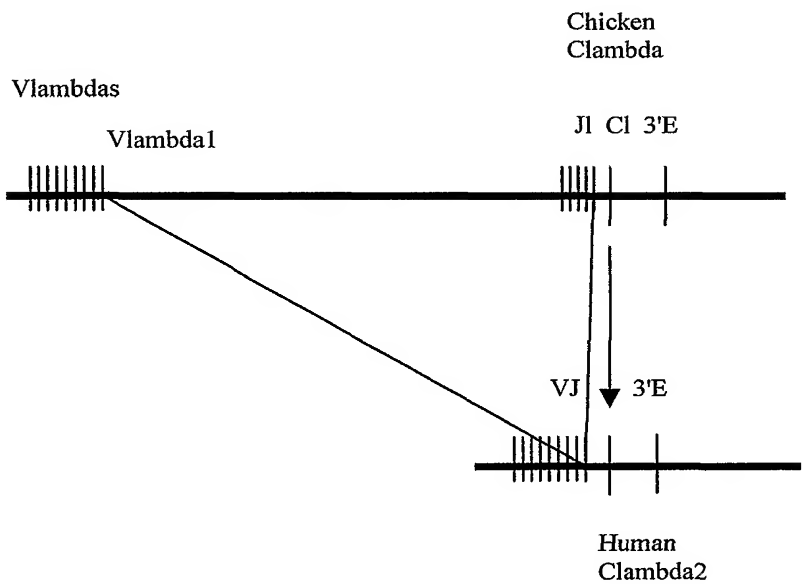

Figure 15. Humanized chicken light chain locus.